From THE DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

POST-TRANSLATIONAL MODIFICATIONS OF MYELIN OLIGODENDROCYTE GLYCOPROTEIN IN CNS AUTOIMMUNITY

Andreas Warnecke

Stockholm, 2016

Cover art:

The cover art was rendered using PyMOL from a model of the sterically optimized MOG_{35-55} epitope presented by H2-IA^b. The epitope features a nitration at Y_{40} (highlighted with red surface) and a MAA adduct on K_{55} (orange surface). The peptide epitope is displayed with black carbons, red oxygens, blue nitrogens and yellow sulfurs. The surfaces are transparent.

I chose this image as it is representative of the respective studies in this thesis, namely by combining elements related to Study I (MDA), Study II (PTMs and modeling), Study III (nitration) and Study IV (antigen presentation by APCs).

Published by Karolinska Institutet.
Printed by AJ E-print AB

ISBN 978-91-7676-345-2

To my beloved family

Post-translational modifications of Myelin Oligodendrocyte Glycoprotein in CNS autoimmunity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Andreas Warnecke

Principal Supervisor:
Prof. Robert A. Harris
Karolinska Institutet
Department of Clinical Neuroscience
Neuroimmunology
Applied Immunology & Immunotherapy

Opponent:
Prof. Christoph J. Binder
Medical University of Vienna
Department of Medical and Chemical Laboratory Diagnostics
Research Center for Molecular Medicine

Co-supervisors:
Prof. Jonas Berquist
Uppsala University
Department of Chemistry
Analytical Chemistry

Prof. Adnane Achour
Karolinska Institutet
Department of Medicine at Solna
X-ray crystallography

Examination Board:
Christina Divne, Ph. D.
Royal Institute of Technology, KTH
Division of Industrial Biotechnology
Structural Biology

Prof. Per-Johan Jakobson
Karolinska Institutet
Department of Medicine at Solna
Rheumatology

Anna Fogdell Hahn, Ph. D.
Karolinska Institutet
Department of Clinical Neuroscience
Clinical Neuroimmunology

Research cannot generate truth, only evidence and conclusions.

ABSTRACT

Autoimmunity towards components of the central nervous system (CNS) is a driving factor of nerve demyelination in Multiple Sclerosis (MS). The etiology of autoimmunity in MS pathogenesis remains elusive, but a combination of genetic predisposition and environmental triggers is theorized to initiate this complex disease.

Oxidative stress and lipid peroxidation represent insults to tissue homeostasis and are associated with inflammation and tissue damage. Reactive molecules associated with these processes covalently modify proteins forming post-translational modifications (PTMs). These PTMs alter the topology and biochemical properties of carrier proteins, which potentially impinges on their recognition by the immune system. Immunological self-tolerance to rare determinants derived from PTM antigen is poorly established, which is a theoretical basis for the development of autoimmunity.

Myelin Oligodendrocyte Glycoprotein (MOG) is a minor surface antigen expressed specifically on myelinating oligodendrocytes in the CNS but is a major target in CNS autoimmunity.

Study I addresses the innate recognition and immunological consequences of MOG modified by malondialdehyde (MDA), a reactive product of lipid peroxidation. MOG-MDA is phagocytosed efficiently and selectively by macrophages via Scavenger Receptor A (SRA), most efficiently by homeostatic M2-type macrophages. Higher uptake of MOG-MDA enhances the proliferation of autoreactive T cells *in vitro* but induces equal severity in experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Taken together the results imply primarily homeostatic clearance of MDA-modified antigen by macrophages, but also a presentation to previously licensed T cells for screening purposes. The phenotype and activation state of the macrophage may influence the response to MDA-modified antigen. Furthermore, the results imply a dissociation of effects mediated by MDA adducts as opposed to the soluble chemical.

Study II introduces a useful *in silico* molecular modeling plugin tool for various PTMs that can be used to address related research questions and predictions.

Study III combines approaches from Study I+II to address the immunology of MOG nitration, a modification related to oxidative stress and inflammation. A combination of molecular modeling, bioinformatics, *in vitro* and *in vivo* data supports the conclusion that nitration denies the presentation of the dominant $MGO_{35\text{-}55}$ epitope by H2-IA^b due to nitration of the critical anchoring residue Tyrosine-40 rendering it incompatible with the p1 pocket. Nitration thus represents a potential silencing effect depending on the major histocompatibility complex (MHC) haplotype. Accordingly, nitrated MOG is poorly encephalitogenic in $H2^b$ C57BL/6 mice, but fully encephalitogenic in $H2^q$ DBA1 mice.

Study IV functionally and phenotypically fully characterizes two fundamentally different sets of bone marrow-derived dendritic cells (BMDCs) generated *in vitro* by alternative protocols. These BMDCs resemble certain populations of antigen presenting cells *in vivo* and can be used to study immunological principles related to immunology.

Collectively, this thesis provides both innovative tools to approach and novel insights relating to the impact of post-translational modifications on autoimmunity.

LIST OF SCIENTIFIC PUBLICATIONS

I. Andreas Warnecke, Sonja Abele, Sravani Musunuri, Jonas Bergquist and Robert A Harris

Scavenger Receptor A mediates the clearance and immunological screening of MDA-modified antigen by M2 type macrophages

Manuscript.

II. Andreas Warnecke, Tatyana Sandalova, Adnane Achour and Robert A. Harris

PyTMs: a useful PyMOL plugin for modeling common post-translational modifications.

BMC Bioinformatics. 2014 Nov 28; 15:370.

III. Andreas Warnecke, Sravani Musunuri, Tatyana Sandalova, Adnane Achour, Jonas Bergquist, Robert A Harris

Nitration of MOG at tyrosine 40 denies epitope presentation via H2-IA^{b} and diminishes encephalitogenicity in an MHC-dependent manner

Submitted manuscript.

IV. Marie N'diaye*[1], Andreas Warnecke*, Sevasti Flytzani, Nada Abdelmagid, Sabrina Ruhrmann, Tomas Olsson, Maja Jagodic*, Robert A. Harris*, André Ortlieb Guerreiro-Cacais*

Rat bone marrow-derived dendritic cells generated with GM-CSF/IL-4 or FLT3L exhibit distinct phenotypical and functional characteristics

Journal of Leukocyte Biology. 2016 Mar; 99(3):437-46. (Epub 2015 Oct 29.)

[1] Asterisks indicate equal contribution; junior or senior, respectively

PUBLICATIONS NOT INCLUDED IN THIS THESIS

V. Sohel Mia*, Andreas Warnecke*, Xingmei Zhang, Vivianne Malmström and Robert A. Harris

An optimized protocol for human M2 macrophages using M-CSF and IL-4/IL-10/TGFβ yields a dominant immunosuppressive phenotype.

Scandinavian Journal of Immunology. 2014 May;79(5):305-14.

VI. Nada Abdelmagid*, Biborka Bereczky-Veress*, Santosh Atanur, Alena Musilová, Václav Zídek, Laura Saba, Andreas Warnecke, Mohsen Khademi, Ana Garcia-Diaz, Anders Hjalmarsson, Elisabeth Aurelius, Marie Studahl, Cécile V. Denis, Tomas Bergström, Birgit Sköldenberg, Ingrid Kockum, Timothy Aitman, Norbert Hübne, Tomas Olsson*, Michal Pravenec*, Margarita Diez*

Von Willebrand factor gene variants associate with *Herpes simplex* encephalitis.

PLoS One. 2016 (accepted manuscript)

VII. Roham Parsa*, Harald Lund*, Ivana Tosevski*, Xingmei Zhang, Ursula Malipiero, Jan Beckervordersandforth, Doron Merkler, Marco Prinz, Alexandra Gyllenberg, Tojo James, Andreas Warnecke, Jan Hillert, Lars Alfredsson, Ingrid Kockum, Tomas Olsson, Adriano Fontana Tobias Suter* and Robert A. Harris*

TGFβ regulates persistent neuroinflammation by controlling Th1 polarization and ROS production via monocyte-derived dendritic cells.

Submitted manuscript.

VIII. Roham Parsa, Harald Lund, Anna-Maria Georgoudaki, Xingmei Zhang, André Ortlieb Guerreiro-Cacais, David Grommisch, Andreas Warnecke, Andrew Croxford, Maja Jagodic, Burkhard Becher, Mikael Karlsson and Robert A. Harris

BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis

JEM, 2016 (accepted manuscript)

Additional ongoing projects with minor contribution.

CONTENTS

LIST OF ABBREVIATIONS

4HNE	4-hydroxy-2-nonenal
AA	Arachidonic acid
ADEM	Acute disseminated encephalomyelitis (pediatric demyelinating disease)
AGEs	Advanced glycation end products
ALEs	Advanced lipoxidation end products
ALU element	Transposable DNA element in primates, originally defined by restriction endonucleases from *Arthrobacter luteus*
ALUM	Aluminium-containing alum vaccine formulation
BBB	Blood brain barrier
BMDCs	Bone marrow-derived dendritic cells
BMMs	Bone marrow macrophages
BTN	Butyrophilin
C57BL/6	(a specific mouse strain)
CCL2	C-C motif chemokine 2 (monocyte attractant)
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant (IFA + *mycobacterium tuberculosis*)
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4 (CD152)
CXCL1	C-X-C motif chemokine 1 (neutrophil attractant)
DA	Dark Agouti (rat strain)
DAMPs	Danger-associated-molecular patterns
DAPI	DAPI (4',6-diamidino-2-phenylindole) (DNA stain)
DBA1	(a specific mouse strain)
DCs	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-3–grabbing nonintegrin (CD209)
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol (reducing agent)
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EF-hand	A structural motif in several calcium binding proteins, named after the involved E and F helices in parvalbumin
ELISA	Enzyme-linked immunosorbent assay
FAAB	2-formyl-3-(alkylamino)butanal
FACS	Fluorescence-activated cell sorting,
FITC	Fluorescein isothiocyanate (fluorescent dye)
FLT3L	FMS-like tyrosine kinase 3 ligand (cytokine important for DC differentiation)
GILT	IFNγ-inducible lysosomal thiol reductase
GST	Glutathione S-transferase
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
i.p.	Intraperitoneal (injection)
i.v.	Intravenous (injection)
IEX	Ion exchange (chromatography)
IFA	Incomplete Freund's Adjuvant (85% (v/v) paraffin oil, 15% (v/v) mannide monooleate
IFN	Interferon

IgV	Immunoglobulin V-set domain (the V indicates the resemblance to the variable domain of antibodies)
IMAC	Immobilized metal affinity chromatography
ISNI	International Society of Neuroimmunology
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAA	Malondialdehyde-acetaldehyde
Mass Spec	Mass Spectrometry
MBP	Myelin basic protein
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
NAWM	Normal appearing white matter
NGF	Nerve growth factor
NIY	Nitrotyrosine
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2; transcription factor
PBS	Phosphate-buffered saline
PE	Phycoerythrin (fluorescent dye)
PGH	Prostaglandin H
pI	Isoelectric point
PLP	Myelin proteolipid protein
PML	Progressive multifocal leukoencephalopathy
Poly I:C	Polyinosinic:polycytidylic acid
PTx	Pertussis toxin
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid Arthritis
RefSeq	NCBI Reference Sequence Database
RT-PCR	Real time polymerase chain reaction
s.c.	Subcutaneous (injection)
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
SRA	Scavenger Receptor A
TCR	T cell receptor
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine substrate
TMP	1,1,3,3-tetramethoxypropane
TNBS	Trinitrobenzenesulfonic acid (used in colorimetric assays to detect amines)
TNF	Tumor necrosis factor
TXA2	Thromboxane A2
Wnt	Wingless type (a protein important for cellular signaling, particularly in development)

1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

1.1.1 THE BASICS OF MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) with recurring autoimmune inflammation directed towards myelin components [1]. MS has an increasing gender bias with up to a 3.5:1 female:male ratio [2]. Scandinavia has the worldwide highest prevalence of MS, almost 2:1000 [3-5], although the overall prevalence is lower at approximately 1:100 000 [2].

Patients typically experience bouts of disease involving a range of neurological symptoms[2] [3] that vary in severity and can be clinically evaluated according to the ordinal Expanded Disability Status Scale (EDSS) [6]. MS is a chronic disease currently lacking a definitive cure. Accordingly, MS can be subdivided into relapsing-remitting (RRMS), secondary progressive (SPMS), or primary progressive (PPMS) courses [7]. The majority of patients presents with RRMS and can be effectively treated for decades, although relapses are not prevented entirely (cf. Figure 1). The increasing disability over time is a serious quality of life issue for patients, and a socio-economic healthcare problem.

1.1.2 THE PATHOLOGY AND MECHANISMS UNDERLYING MS

1.1.2.1 Myelin

In order to understand MS we need to understand myelin. Briefly, myelin is the insulation of nerve axons that allows for salutatory[3] conduction of signals mediated by depolarizing action potentials. The myelin insulation organizes various sodium channels to intermitting unmyelinated sections known as nodes of Ranvier. Signal transduction 'jumps' along these nodes, instead of moving along the entire axon at a significantly slower rate [8-10].

Importantly, axons do not necessarily require myelination to function independently[4], although myelination is required for fast signal transduction. Accordingly, myelinated axons grow highly dependent on trophic support by the myelinating cell, especially since myelination isolates axon stretches from access to the interstitial space [8]. Axon myelination is performed by Oligodendrocytes in the CNS, and by Schwann cells in the periphery [9]. Apart from tissue

[2] Among others: numbness, vision disturbances, pain, depression, urogenital problems, spasticity, fatigue, cognitive impairment, paralysis and ataxia.

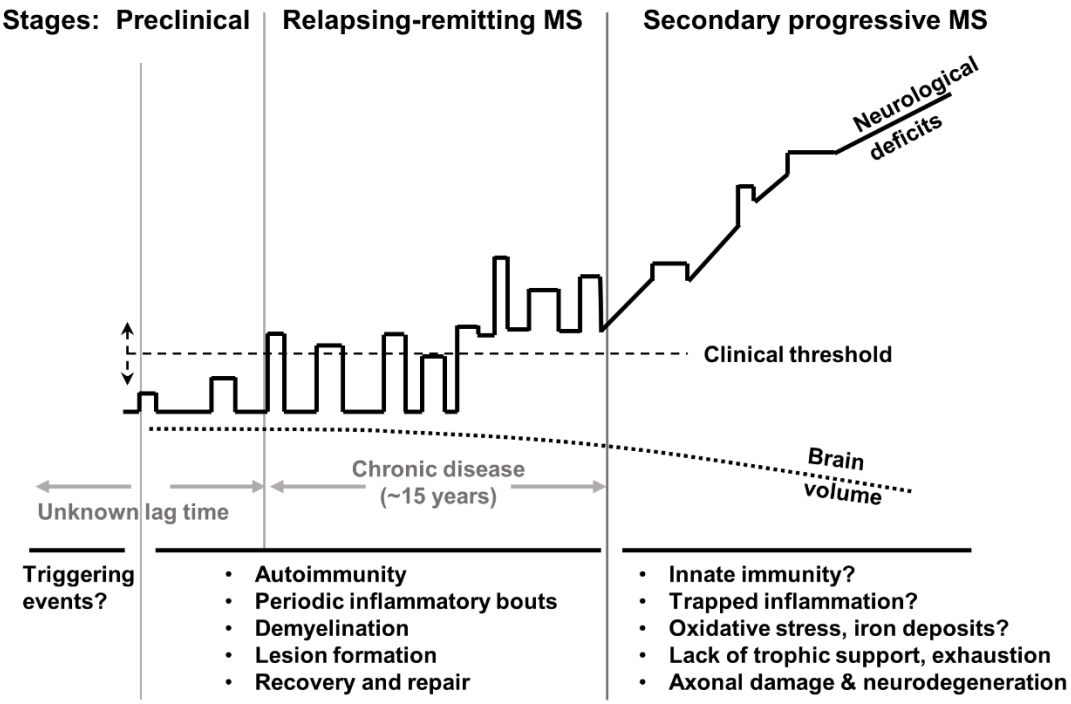
[3] Latin: *saltare*: jump

[4] As evident by low conductivity, unmyelinated C fibers

specificity, there are notable differences between these cell types: Schwann cells only myelinate one stretch of a single axon, while Oligodendrocytes myelinate multiple axons [9]. Importantly, there are several Oligodendrocyte-specific proteins, including PLP, MBP, CNP and MOG (Figure 2).

The biology of myelination is extremely fascinating and too complex to be discussed here in full detail (cf. [8-10] for reviews). I recommend reading references [8, 9], which contain brilliant illustrations. The bottom line is that the entire myelin machinery is devoted to metabolically support the axon, which in turn becomes highly efficient, but also entirely dependent on the performance of the myelinating cell.

Figure 1: Schematic course of Multiple Sclerosis



The triggering events initiating Multiple Sclerosis (MS) precede clinical presentation by an unknown period of time, presumably several years. Preclinical autoimmune inflammation and lesion formation possibly occur long before noticeable manifestation, with variability in the individual's threshold. Clinically evident relapsing-remitting MS is further characterized by bouts of disease and recovery with variable frequency and intensity. This course may last decades with the available disease modifying drugs that reduce the relapse rate. Over time, however, neurological deficits and brain atrophy increase and are not fully resolved.
In progressive MS, which is as yet driven by not fully elucidated alternative mechanisms, the classical treatment options do not yield benefit. Neurological deficits persist, most likely due to irreversible axon damage and neurodegeneration as the CNS fails to compensate for demyelination and the lack of trophic support.
It is debated whether secondary and primary progressive MS share the same late stage mechanisms but differ in the time of clinical manifestation. The figure was adapted from (Olsson, 2014), after an original conception by Mohsen Khademi.

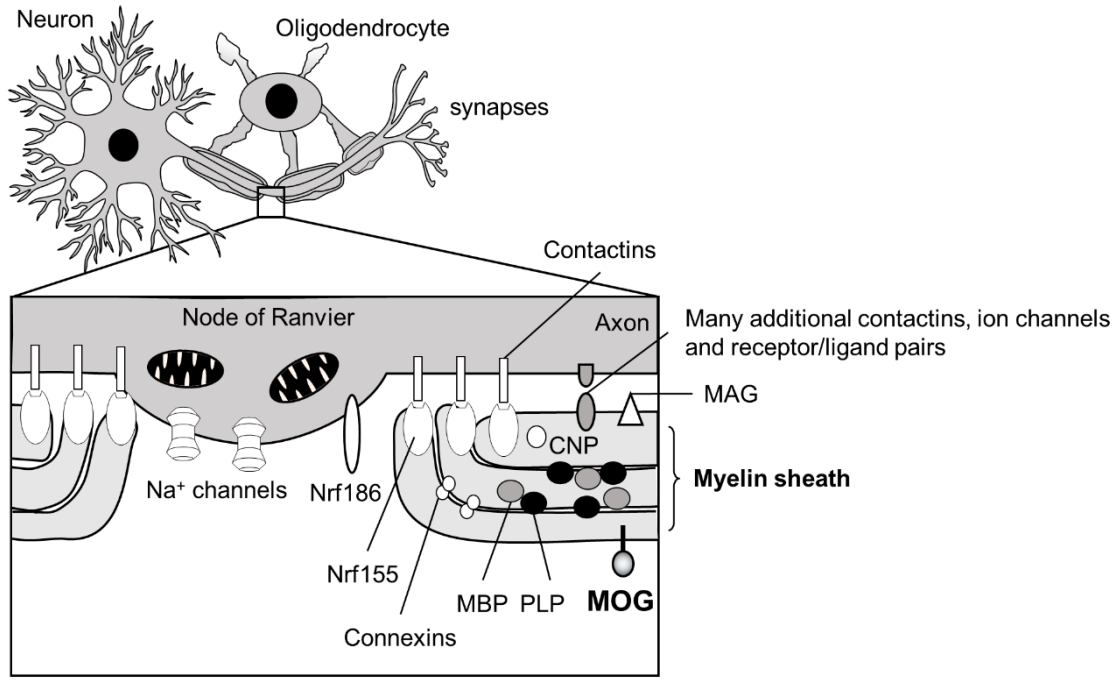
1.1.2.2 Demyelination and MS pathology

Tus far we have established the importance of trophic support for axons by myelinating oligodendrocytes [8]. In MS this dependence becomes disturbed as myelin becomes focally destroyed by the misguided immune system. Hallmarks of MS pathology include inflammation, temporally and focally dynamic de- and remyelination, complement deposition, compromised blood-brain barrier integrity, tertiary lymphatic follicles in the CNS, brain atrophy, axonal damage and neurodegeneration [1, 11].

Though demyelination is in principle reversible it presents a major insult to the homeostasis of the CNS, which is one factor underlying the relapsing-remitting phenotype of MS [12]. Even axonal damage can be temporary [13], but the level of stress may vary depending on whether the axon has access to interstitial space for autarkic support or whether it is being 'suffocated' by malfunctional myelin. Proficient remyelination is required for a return to homeostasis, but it is the irreversible degree of neurodegeneration that correlates with disability in MS [1, 11].

The pathogenesis of MS is very complex (reviewed in [1]). Notably, most MS mechanisms are being unraveled based on translation of the EAE animal model. Relevant mechanisms and the EAE model will be discussed further below.

Figure 2: Myelin structure



The inlet magnifies a half section of a myelinated CNS axon near the node of Ranvier. Selected proteins and their subcellular locations are depicted. The figure was adapted from Mayer *et al.* (2012).

CNP: 2',3'-cyclic-nucleotide 3'-phosphodiesterase (3rd most abundant myelin protein)
MAG: Myelin Associated Glycoprotein
MOG: Myelin Oligodendrocyte Glycoprotein
MBP: Myelin Basic Protein (2nd most abundant myelin protein)
Nrf: Neurofascin
PLP: Myelin proteolipid protein (most abundant myelin protein)

1.1.2.3 T cells as main mediators of disease in MS

A ground laying genome-wide association study for MS has confirmed a role for inflammatory cell-mediated disease [14]. The initiation of MS itself is incompletely understood [15], but the prerequisite is that autoreactive lymphocytes escape deletion by central tolerance mechanisms. In fact, MS is largely driven by autoreactive T cells [16], also evident by the effect of treatments targeting T cells [17] (discussed further blow). Taken together, this indicates underlying defects in the maintenance of tolerance mechanisms [18].

A plethora of studies has since unraveled mechanistic differences of Th1 and Th17 cells in CNS inflammation, along with the relative importance of various cytokines [19, 20]. In order for T cells to be licensed they rely on MHC-restricted antigen being presented by various activated APCs (reviewed in [21]). In this context, antigen processing and presentation are vital

[22], as are effects such as bystander activation. It has recently been suggested that the lungs are possibly an organ where T cells may acquire a CNS-inflammatory phenotype [23]. The current understanding is that T cells, which recognize various myelin-specific epitopes, transgress into the CNS and induce inflammation. The demyelination itself appears to be orchestrated by T cells but is specifically mediated by infiltrating GM-CSF-driven monocytes [24, 25], whereas CNS-resident microglia clear debris [26]. Notably, in human MS there is a significant contribution by $CD8^+$ T cells, which is poorly represented in most EAE models [27].

Furthermore, epitope spreading to new determinants can occur over the course of CNS inflammation [28, 29]. This epitope spreading is relevant for the propagation of autoimmune inflammation and induction of relapses [12]. Taken together, both in patients and EAE, there is ample evidence for individual T cell responses against myelin antigens such as PLP, MBP and MOG [1].

1.1.2.4 B cells and autoantibodies in MS

Although MS is perceived to be mainly T cell mediated, B cells and antibodies contribute significantly to the pathology [30]. Tertiary lymphoid follicles and oligoclonal immunoglobulin bands inside the CNS are hallmarks of CNS inflammation in MS, and furthermore the CNS entertains a B cell fostering milieu [1, 30]. The relevance of B cells in MS is highlighted by the fact that several B cell-targeting treatment are effective in RRMS [30].

The contribution of B cells to MS is two-fold: first, B cells secrete pro-inflammatory cytokines such as IL-6 [31] and act as APCs independently of their capability to produce secreted antibodies [32]. Secondly, B cells and differentiated plasma cells are the producers of auto-antibodies against various CNS antigens [30], including MOG [33], Neurofascin 155 [34] and possibly KIR4.1 [35].

The most-studied autoantigen in MS is MOG. This relates to the fact that MOG is a major antigen in myelin, despite being poorly represented (0.05% of myelin protein) [36] and its location in the outermost layer of myelin, as opposed to other myelin components (cf. Figure 2). However, the anti-MOG seropositivity for MS is low (<4%), and more prevalent in pediatric demyelinating diseases such as ADEM[5] (~27%) (reviewed in [37]). Depending on the study, up to ~30% of MS patients tests positive for MOG antibodies [38] nonetheless, but there is significant heterogeneity in approaches and study populations [37]. The method and material used to test for autoantibodies notably have significant impact on the readout [33]. For instance, there is a significant amount of human antibodies that are not cross-reactive with rodent MOG, as tested with various MOG-expressing transfected cells [39]. Furthermore, MOG glycosylation [40] and conformation [41] are factors that potentially affect its recognition by

[5] acute disseminated encephalomyelitis (ADEM)

antibodies. Accordingly, cell-based assays are suggested to be most effective [37]. The heterogeneity of MS patients in terms of antibody responses, and the lack universal testing methods, assay cutoffs, the possibility of intrathecal local production and lastly the fact that even healthy controls are detected with MOG antibodies severely limit the usefulness of MOG as a diagnostic marker [33, 37, 42]. However, MOG seropositivity in MS patients possibly offers a stratification principle, e.g. for B cell targeting therapies [42].

A novel axonal autoantigen was recently reported with a subgroup of MS patients having antibodies towards the potassium channel KIR4.1 [43, 44][6]. Such KIR4.1 antibodies were subsequently implicated in the pathology of MS [35] and childhood acquired demyelinating disease [45]. However, several failures to independently confirm KIR4.1 as an autoantigen have contributed to a highly controversial state of the subject [46][7] [47]. Possibly technical approaches are to blame for these discrepancies: first, the detection relies on peptide ELISAs in many experiments, one of the sequences reportedly requiring chemical 'stapling' in order to be a conformational epitope[8]. Secondly, with regard to the endogenous protein, that lab proposes that neuron-specific glycosylation of KIR4.1 may be required for antibody recognition[9]. Personally I am skeptical towards both these claims as they are somewhat contradicting. Furthermore peptide ELISAs for conformational epitopes are technically highly questionable. Much credibility is given to the group's report that they could identify MS patients in blinded samples using KIR4.1 reactivity as a marker[10]. Whether KIR4.1 as an MS autoantigen withstands long-term scrutiny will have to be evaluated.

1.1.2.5 Progressive MS

There are essentially two forms of progressive MS: secondary or primary progressive.[11] SPMS can be regarded as the inevitable continuation of RRMS when remissions cease to occur and treatments lose their effect. Conversely, PPMS variants are regarded as aggressive forms and typically lack signs of improvement.

The pathological hallmarks of progressive MS are shared with RRMS but are often more pronounced [11]. The current hypothesis implies that progressive MS is driven by additional pathological mechanisms [11]. These include compartmentalized CNS inflammation, oxidative damage (enhanced by extracellular iron deposition), mitochondrial dysfunction, diffuse white matter injury, glutamate-induced excitotoxicity and failing trophic support, all of which lastly contribute to axonal degeneration and irreversible neuronal death [8, 11, 48, 49].

---

[6] ATP-dependent inwardly rectifying potassium channel Kir4.1; (K = potassium, ir = inward rectifying)
[7] This report failed to detect significant differences, despite collaborative approaches with the lab originally reporting Kir4.1 as an autoantigen. Many additional labs criticized the validity of KIR4.1 as autoantigen in MS (ISNI conference in Mainz, 2014).
[8] ISNI conference in Mainz, 2014; personal communication with Rajneesh Srivastava (first author of the original KIR4.1 report)
[9] As above, with poster and abstract
[10] Bernhard Hemmer (last author of the original KIR4.1 report), ISNI 2014 talk
[11] There are more variants distinguished clinically that I decided to omit

1.1.3 RISK FACTORS

While the exact etiology of MS is still elusive it is unrealistic that a single cause will ever be identified because MS is a complex heterogeneous disease [50] – a syndrome with many causes rather than a single disease entity. Though the interplay of various risk factors is poorly understood, it is commonly accepted that the cumulative stacking of individual risks beyond a certain threshold is responsible for initiating MS as a result of genetic predisposition and environmental triggers.

1.1.3.1 Genetic risk factors

The strongest genetic association with MS is the HLA region [51]. Specific haplotypes are associated with increased risk of MS[12], in particular the class II HLA-DRB1*15:01 allele with an odds ratio of 3.92 (8.30 if homozygous) [51].

In contrast to the Rheumatoid Arthritis (RA) field, in which HLA risk haplotypes associated to RA and their respective (modified) antigens are mostly resolved [52], the presented epitopes in MS are still elusive. Identifying potential target antigens may go a long way in understanding more about the pathogenesis of MS. Conversely there is the notion that certain HLA alleles may exert antigen-independent effects. It is also puzzling that there are numerous class I alleles that display a protective effect against MS[13], notably HLA-A*02:01. How this relates to the risk and pathogenesis of MS remains unclear, but a connection to viral clearance or deletion of autoreactive $CD8^+$ T cells appears plausible.

Remaining non-HLA genes are more complex. The large scale GWAS study [14][14] revealed a long list of candidate MS risk genes, both previously known and novel ones. The majority of those strongly supported an involvement of the immune system in MS, in particular T cells. Importantly, the individual odds ratios of these non-HLA genes are comparatively low, ranging from just over one to ~1.6 at the most. In parallel, animal congenic strains have been useful in identifying loci that regulate EAE susceptibility [53].

A future development already in progress will be to elucidate the role of identified candidate genes in MS risk and pathogenesis, but also to investigate potential interactions and the combined risk of multiple parameters, both genetic and environmental [54].

1.1.3.2 Environmental risk factors

The concept of MS as a complex disease predates the recent genetic studies and several environmental factors have been implied in the risk of MS [55].

---

[12] Class II risk alleles: HLA-DRB1*15:01, HLA-DRB1*13:03,HLA-DRB1*03:01, HLA-DRB1*08:01, HLA-DQB1*03:02; Positive interactions: HLA-DQA1*01:01 with HLA-DRB1*15:01; HLA-DQB1*03:01 with HLA-DQB1*03:02 (Moutsianas *et al.*, 2015)

[13] Class I protective alleles: HLA-A*02:01, HLA-B*44:02, HLA-B*38:01, HLA-B*55:01 (Moutsianas *et al.*, 2015)

[14] Make sure to check the supplementary information of this article for a detailed list of candidate genes.

Major environmental factors associated with MS include smoking [56], lack of vitamin D and infection with Epstein-Barr virus, particularly adult infectious mononucleosis [21][15, 57]. Other confirmed risk factors for MS include obesity [58] and night shift work for young adults [59]. Dysregulation of N-glycosylation has been proposed as a mechanism potentially driving MS [14] and sequence identity of MOG R_{101}DHSYQEE$_{108}$ and C*hlamydia* was suggested as molecular mimicry [60][15]. However, the latter two concepts have not been validated or experimentally confirmed.

The lack of vitamin D has been associated with elevated MS risk based on a latitudinal distribution of MS: prevalence is more common with distance from the equator, even within Sweden [5]. The latitudinal distribution of MS was attributed to lack of 1,25-dihydroxyvitamin D, precursors of which are generated in the skin in response to sunlight [15, 55, 61]. Notably, it has been demonstrated that this effect takes effect in early life [62, 63]. Multiple experimental studies have since demonstrated effects of vitamin D on immune system elements including DCs, macrophages and T cells [61], particularly Th17 cells [64, 65], and amelioration of EAE [66]. Another line of evidence is seasonal variation of relapses, most likely connected to the expression of immune-related genes [67]. MS patient self-reportedly tend to fair better with sunlight exposure and milder climate, but also with physical exercise[16]. Among other studies, the exercise aspect has been addressed in the EAE model with positive implications: mice with voluntary access to a running wheel exhibited milder EAE symptoms [68]. On the other hand it is unclear to which degree genetic effects influence the geographical distribution of MS. Furthermore, UV radiation has effects that are vitamin D-independent [69].

Cigarette smoking is by far the largest, and potentially avoidable, risk factor not only for MS, but for autoimmune diseases in general [56]. The mechanisms through which smoking drives autoimmunity are still elusive, but likely relate to depleted antioxidant responses and chronic immune activation in the lungs, recently demonstrated as being very relevant to T cell licensing [23]. Post-translational modifications promoted by reactive components potentially contribute to inflammation, but detailed studies will be required to address these notions in the context of EAE. Interestingly, epidemiological studies have revealed that oral tobacco 'snus' has protective effects on MS [70][17]. This implies nicotine as a candidate immunomodulatory mediator [71]. Importantly, there is positive interaction of the MS risk for smoking and risk HLA haplotypes [72]. This interaction is highly relevant in the context of genetic predisposition and environmental triggers conveying a stacking overall risk of developing MS.

---

[15] This sequence happens to localize to the DA rat (1AV1 haplotype) MOG epitope 91-108, although this could possibly be random; the RDHSYQEE sequence is very short and reminds more of a CD8/ MHC class 1 epitope. *Chlamydia spp*. are intracellular parasites.

[16] Patient meeting during courses.

[17] Prof. T. Olsson from our Neuroimmunology Unit frequently takes the opportunity to highlight this research result to the entertainment of the audience

1.1.4 TREATMENT OF MS

For a more complete and detailed perspective on the treatment of MS I refer to a recent review by F. Piehl [17], a clinician from our department.

Current first-line treatments mostly employ IFNβ (1a: Avonex, Rebif; Plegridy[18] [7, 73]; 1b: Betaseron/betaferson, Extavia,) or glatiramer acetate (Copaxone[19]). IFNβ has multiple effects on T cells and neuronal survival (reviewed in [74]), and has been reported to inhibit the differentiation of Th17 cells [75]. Interestingly serum IL7 has been suggested as a marker for treatment benefit with IFNβ [76]. The mechanism of glatiramer acetate is elusive, but it may affect antigen presentation and has been reported to promote Tregs.

Infusions of monoclonal antibodies have the benefit of being highly specific for the target they were raised against and, unlike small molecules, do not carry the same risk of various metabolic or off-target side-effects. However, antibodies may not have the same permeability and their exogenous nature carries a risk of patients developing intolerance to the antibody. The current trend is therefore to strive towards humanized, or entirely human antibodies to maximize their safety profile. A major healthcare challenge is that these antibody preparations are extremely expensive.

Natalizumab[20] is successfully used in the treatment of MS under the trade name Tysabri. Its target it the Integrin alpha-4, part of the VLA-4[21] complex on lymphocytes, which is important for their migration into CNS tissue. Although Tysabri is very effective in treating RRMS, a clinical problem is posed by the risk of developing progressive multifocal leukoencephalopathy (PML) as a result of opportunistic infection with JC virus[22] which is no longer controlled by virus-specific T cells affected by treatment [17, 55]. This complication requires monitoring patients for JC virus and switching treatment regime, but also highlights the challenges arising from targeting immune mechanisms indiscriminately.

Other antibodies for specific treatment, both existing and in development, are Rituximab[23], Ocrelizumab[24] and Ofatumumab[25] (all anti-CD20 on B cells), Daclizumab[26] (anti-CD25 on T cells) and Alemtuzumab[27] (anti-CD52 on lymphocytes) [7, 17].

Fingolimod (Gilenya) is an orally-administered small molecule antagonist to sphingosine-1-phosphate receptors (S1PRs) on lymphocytes and prevents the egression of T cells into the

[18] A PEGylated version with longer half-life requiring fewer administrations
[19] A mixture of randomized oligomers of amino acids in MBP
[20] Tysabri, humanized monoclonal
[21] Very late antigen 4
[22] John Cunningham virus
[23] Rituxan, mouse/human chimeric monoclonal
[24] *Pending tradename*, humanized monoclonal
[25] Arzerra; pure human monoclonal,
[26] Zenapax, humanized monoclonal
[27] Lemtrada, humanized monoclonal; originally designed as a lymphoma treatment

circulation. Other oral drugs are Teriflunomide (Aubagio) that targets cell proliferation, and Laquinimod (Nerventra) that modulates NF-κB [17].

An upcoming novel drug is dimethyl fumarate (DMF, BG-12, Tecfidera). Despite being successfully tested in clinical trials [77, 78] its mode of action is elusive, but one suggestion is that DMF activates the transcription factor Nrf2 and the cellular antioxidant response [17]. The activation of Nrf2 most likely can be attributed to the ability of DMF to undergo Michael additions [79] with primary amines, thus forming protein adducts that possibly deactivate Keap1[28], a negative regulator that sequesters Nrf2 in the cytoplasm and promotes its degradation. There are valid concerns related to the long term efficiency of DMF [80] and the fact that it possibly evokes severe allergic skin reactions [81]. I am bringing up the latter because it relates to protein adduction and MDA.

My concluding perspective on existing treatments is that they target various immune mechanisms related to CNS inflammation. Based on the new mechanisms identified in preclinical models we may expect novel targets already being evaluated [7]. Importantly, there is the need to elucidate the mechanisms driving progressive MS and to identify potential interventions. Neuroprotection, myelin repair and oxidative balance are emerging aspects that will be of interest in this context [1, 48, 82]. Secondly, early diagnostic markers would be valuable to diagnose MS early and to start treatment early or possibly to prevent disease development to begin with.

1.1.5 CONCLUDING REFLECTIONS ON MS

In summary, there is ample evidence for MS being an inflammatory autoimmune disease entailing demyelination and neurodegeneration. While the etiology and mechanisms of MS are still elusive we begin to understand vital pieces of the puzzle. Future MS research will be dedicated to solving the outstanding questions and identifying the contribution of individual risk genes, environmental factors and their interplay. This may reveal new leads for potential interventions, especially with the aim of curing the cause and halting progression. Understanding the recognized heterogeneity among MS patients and disease courses will be vital in the quest for resolving disease mechanisms, but also for diagnosis and treatment stratification.

[28] Kelch-like ECH-associated protein 1

1.2 MYELIN OLIGODENDROCYTE GLYCOPROTEIN

1.2.1 THE MOG GENE

Interestingly, the gene locus for Myelin Oligodendrocyte Glycoprotein (MOG) is located within the HLA region. The HLA region located on chromosome 6p in humans and on chromosome 17 for mice is otherwise well known for its abundance of genes related to the immune system, in particular the HLA genes involved in antigen presentation. However, MOG itself appears not to fall into that category. Importantly, the expression of MOG is restricted to the CNS in mature oligodendrocytes, late in their maturation process [36].

In the human genome the MOG gene spans around 17 kb and has 8 exons [83]. Notably these exons undergo complex alternative splicing in humans but not in rodents [84]. On a side note, complex alternative splicing in primates is in general believed to a major factor accounting for an elevated evolutionary complexity in the light of otherwise striking similarity between orthologous genes [85]. Conversely, alternative splicing has been suggested to be a factor contributing to the development of autoimmunity [86]. One underlying principle for the latter is a lack of immune tolerance as a consequence of targeted regions of autoantigens being encoded by exons that are not expressed during embryonic development or especially in the thymus mediating the deletion of autoreactive T cells. A prime example is that immunodominant epitopes of PLP map to a region that is absent in its shorter isoform (called DM20), which is the only variant expressed in the thymus unlike in mature oligodendrocytes [22, 86].

The current USCS genome browser assembly[29] [87] lists 9 curated human MOG isoforms[30] and additional variants pending RefSeq validation can be accessed[31]. By comparison the mouse assembly[32] features two variants, one with all exons and the other lacking exon 6. The rat assembly[33] only lists one variant. The respective splice variants have been described in detail by Delarasse *et al.* [85], and I have included an adapted summary in Figure 3. Nonetheless, the splicing of MOG itself is considerably complex and the respective splice variants that affect mostly the C-terminal, intracellular domains of MOG and are discussed below.

[29] Dec. 2013 (GRCh38/hg38)
[30] uniprot.org lists 9 curated isoforms and a total of 13 isoforms
[31] XM_005249139.2 for a transcript containing the ALU element, corresponding to a secreted variant of MOG
[32] Dec. 2011 (GRCm38/mm10)
[33] Mar. 2012 (RGSC 5.0/rn5)

1.2.2 THE MOG PROTEIN STRUCTURE

1.2.2.1 MOG protein domains and features

Discussing MOG gene architecture goes hand-in-hand with the corresponding protein structure. The exons are as follows: Exon 1 = signal peptide (removed during processing), Exon 2 = extracellular IgV domain, exon 3= transmembrane domain, exons 4 & 5 = cytoplasmic regions, exon 6A/B = membrane associated region, exon 7 = linking region, exon 8A/B = tail region. Additionally, the so-called ALU element in intron 2 is considered a non-canonical alternative exon that encodes less-abundant, truncated and soluble versions of MOG. The individual domains are depicted in Figure 3.

Importantly, the canonical and most abundant isoform of MOG, namely α1, corresponds to the rodent versions and consists of the extracellular domain (exons 1+2) followed by a single transmembrane domain (exon 3), a cytoplasmic stretch (exons 4+5), a membrane-associated region (exon 6) and a tail region (exons 7+8) [84, 88], cf. Figure 3.

The MOG protein was originally discovered as a lectin-binding (i.e. glycosylated) protein [88][34] purified specifically from CNS tissue. MOG was further characterized by its lacking resemblance of known CNS proteins such as Myelin Basic Protein (MBP) and lastly defined as the target recognized by the monoclonal antibody 8-18C5 [89]. Of all historic alternative names Myelin Oligodendrocyte Glycoprotein (MOG) was established in recognition of the glycosylation, specific expression in oligodendrocytes and association to myelin [36].

1.2.2.2 The extracellular IgV domain of MOG

Most research focus has addressed the conserved extracellular domain of MOG. The crystal structure of recombinant MOG has been resolved for rat MOG alone [PDB-ID: 1PKO] (1.45 Å) and as a complex of rat MOG with F_{ab} fragments of the defining anti-MOG antibody 8-18C5 [PDB-ID: 1PKQ] (3.0 Å) [60]. Furthermore, the crystal structure of recombinant mouse MOG has been resolved independently [PDB-ID: 1PY9] [90]. This extracellular domain of MOG belongs to the immunoglobulin V-set (IgV) as it is structurally related to the variable domain of antibodies [60, 90]. The IgV domain features the typical antiparallel β sheets, linked by a single disulfide bridge between the B and F sheets (cf. Figure 3).

Intron 2 contains the so-called ALU element that can serve as a non-canonical exon with two possible splice variations both containing a stop codon, named ALU-10A or ALU-10B, respectively [84] (cf. Figure 3). Furthermore, transcription of variants containing the 2' intron

[34] It was later shown that some isoforms are not glycosylated and insensitive to the glycosidase Endo-H

region constitutes a third alternative stop codon, yielding the Ig-2' transcript of MOG [84]. These three variants are considered to be secreted versions of MOG, consisting of essentially only the IgV domain, yet their biological role or relevance remain unknown.

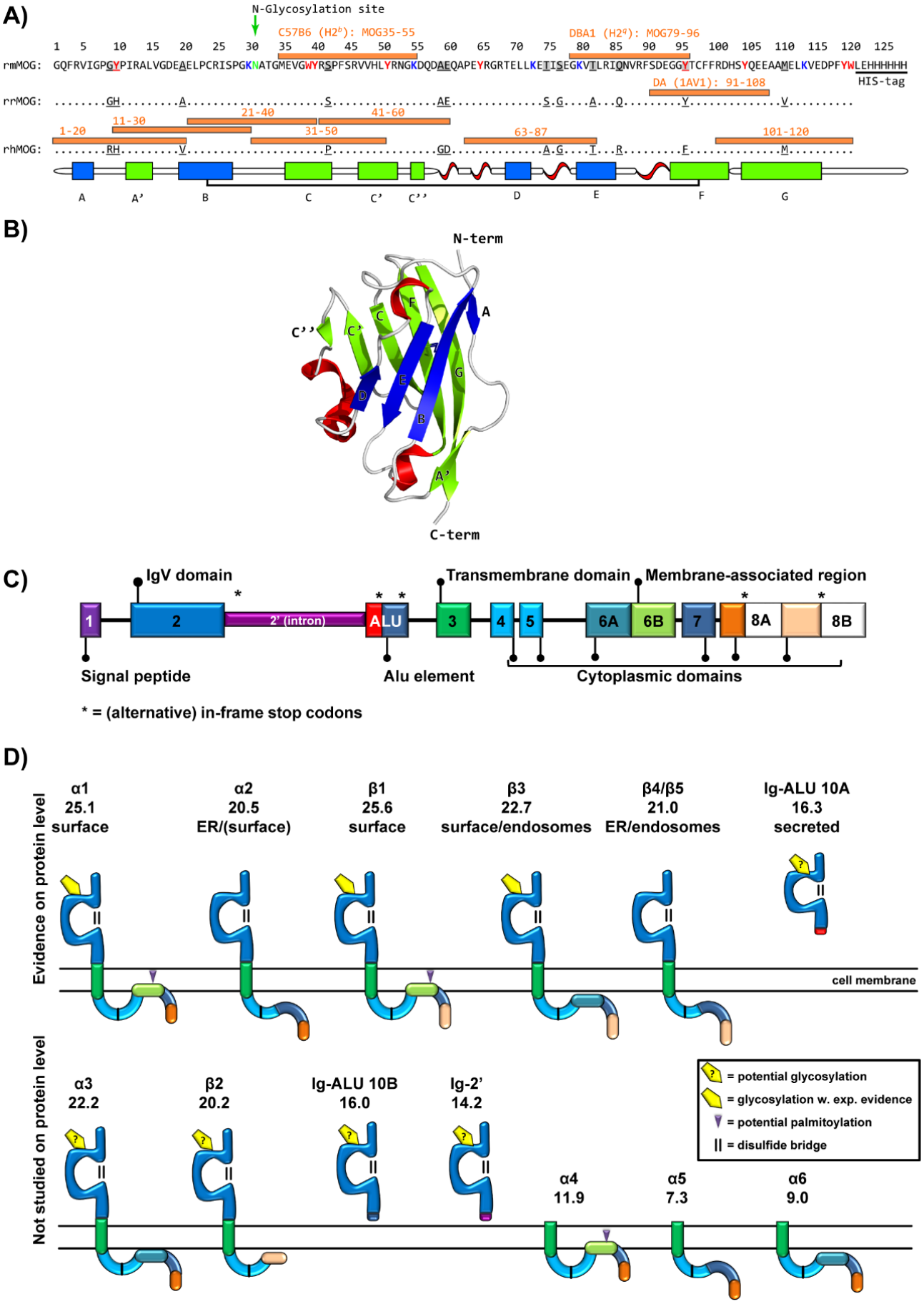
The IgV domain supposedly mediates interaction with putative ligands and thereby the function of MOG. Although the overall importance and exact function of MOG remains elusive, studies have proposed a range of potential functions including MOG being a homo-dimer and adhesion molecule [90], MOG regulating the classical complement pathway [91] or MOG being a receptor for Nerve growth factor (NGF) [92]. These functions will be discussed individually below.

1.2.2.3 The cytoplasmic domains of MOG

Very little is known about the function of the cytoplasmic domains of MOG, apart that the isoforms influence the cellular localization, to e.g. the surface or endoplasmic reticulum [88]. Especially in rodents, MOG is mainly regarded as a surface-expressed protein with a single transmembrane domain. Interestingly, exon 6A encodes a stretch of the membrane associated domain containing two cysteines C177 and C198[35] that are hypothesized to either be palmitoylated and/or interact with membrane galactocerebroside (Gal-C), the latter being a specific glycosphingolipid marker for oligodendrocytes [36]. Palmitoylation and interaction with Gal-C would support that this domain interacts closely or alternatively is embedded into the cell membrane. Exon 6B is by contrast devoid of cysteines. Nonetheless, the functional consequences of cytoplasmic variations, potential interacting proteins and possibly signaling pathways associated to MOG are yet to be identified. In particular the existence and relevance of variants lacking the extracellular domain requires clarification.

[35] Numbering according to processed MOG sequence

Figure 3: Myelin Oligodendrocyte Glycoprotein



A) Sequences and structure information for recombinant MOG: Top: sequences of recombinant mouse MOG (rmMOG), rat MOG (rrMOG) and human MOG (rhMOG); Lysines are indicated in bold blue, Tyrosines or Tryptophanes in bold red; selected epitopes in EAE and MS are indicated with orange bars, the MHC haplotype is indicated in brackets for the rodent strains. Bottom: The secondary structure and nomenclature, as adapted from (Clements *et al.*, 2003): white = loops, red = α-helix, glue/green = proximal/distal β-sheet (cf. B). The connecting line between the B and F β-sheets indicates the disulfide bridge.

B) 3D model of rmMOG from [PDB-ID: 1PY9] (Clements *et al.*, 2003). The colors and nomenclature correspond to those in in A.

C) The gene structure of MOG. Exons and ALU element = thick bars, introns = thin bars; Intron/exon lengths are indicative of size, but not to scale. The protein domains encoded by the corresponding exons are labeled as indicated. Exons 6 and 8 feature mutually exclusive A or B splicing alternatives. Adapted from (Delarasse *et al.*, 2006).

D) Simplified illustration of MOG proteins corresponding to the indicated splice variants. Note that the colors correspond to the exons in C). The predicted size [kDa] is displayed beneath the splice variant name. Additionally the cellular location is indicated for experimentally-studied variants (Boyle *et al.*, 2007). Potential, natural post-translational modifications (glycosylation, Palmitoylation; cf. Johns *et al.*, 1999) are depicted as in the legend insert. Note that exon 6A contains modifiable cysteine residues (C177 and C198), whereas exon 6B does not (cf. Uniprot-ID: Q16653, α1 vs. α3). The membrane-associated region encoded by exon 6 is depicted as membrane-embedded for exon 6A and cytoplasmic for exon 6B.

1.2.3 STRUCTURAL SIMILARITY OF MOG TO THE B7 FAMILY AND BUTYROPHILINS

The IgV domain of MOG has significant structural similarity to members of the B7 family such as CD80, CD86 and PD-1. CD80 and CD86 bind the activating CD28 receptor on T cells or the attenuating CTLA-4 on activated T cells. However, although the tertiary structure of the MOG IgV domain is strikingly similar to that of CD80, MOG does not feature the amino acid topology that would allow binding to either CD28 or CTLA-4. This statement is based on a series of structural alignments I performed using the crystal structures of MOG [PDB-ID: 1PY9], CD28 [PDB-ID: 1YJD] and a complex of B7.1 (mouse CD80) and CTLA-4 [PDB-ID: 1I8L] (data not shown).

Furthermore, the MOG IgV domain shares structural similarity to domains of butyrophilin (BTN) family. Butyrophilins have diverse tissue-specific roles related to lipid secretion and metabolism. In particular, BTN1A1 has established roles in the mammary gland and is present in milk lipid droplets [93]. Other more ubiquitous functions relate to immunology: BTN1A1 and BTN2A2 have been demonstrated to negatively regulate T cell activation and cytokine secretion [93]. However, MOG was used as a control in the study identifying a T cell regulatory role for BTN1A1 and BTN2A2 [93], but had no such effect. BTN3A (CD277) has a role in the

activation of γδ T cells [94], which is interesting given that γδ T cells may contribute to MS [95].

Interestingly, the similarity of MOG to butyrophilins present in milk has inspired the hypothesis that dairy-containing diet may influence the risk or treatment of MS. Immunization of DA rats with BTN_{74-94} peptide or passive transfer of BTN_{74-94}-specific T cells evoked meningeal and perivascular cell infiltrates in the CNS [96]. Conversely, high-dose i.v. treatment with BTN_{74-94} peptide ameliorated EAE induced by MOG_{74-94}-specific T cell transfer [96]. Another study reported antibody cross-reactivity between BTN and MOG in MS patients and suggested molecular mimicry as an underlying principle [97]. Regrettably this particular study is entirely based on antibody ELISA using peptide epitopes as opposed to folded protein.

Highlighting the structural similarity of MOG to proteins that happen to have roles in immunology is misleading in a sense that it may prompt the assumption that MOG itself may have such a function. However, I should point out that the immunoglobulin superfamily has many members with functions not related to the immune system and that the name is mostly historical and relates to the structural features of antibodies, a.k.a. immunoglobulins. Similarity in tertiary structure alone does not allow the drawing of conclusions about function.

1.2.4 THE PHYSIOLOGICAL FUNCTIONS OF MOG

The function of MOG is either redundant or non-essential. Firstly, MOG appears relatively recently in evolution and can be found in mammals, but is absent in other vertebrae e.g. reptiles [36]. Secondly, MOG knockout mice display no physiological phenotype [98].

1.2.4.1 MOG as an adhesion molecule

One proposed function for MOG is as an adhesion molecule for myelin compaction. First, MOG shares sequence homology with other adhesion molecules, namely the adenovirus receptor D1 domain (CAR), myelin protein zero (P_0) and junctional adhesion molecule (JAM) [90]. Secondly, mouse MOG has the tendency to form dimers resistant to denaturing conditions *in vitro* [36, 90]. Dimerization of MOG is supported independently by biochemical evidence [36], Biacore measurements [90], as well as structural modeling of the MOG dimer [90]. However, what argues against MOG being an adhesion molecule is the fact that MOG represents a meagre 0.05% of myelin protein [36], making potential interactions statistically rare. Furthermore, even ectopic expression in cell lines did not induce cell clumping, in contrast to P_0 [36]. Lastly, electron microscopy comparing myelin from wild type and MOG knockout mice did not reveal notable differences [98]. Nonetheless, the possibility that MOG serves as a minor adhesion molecule cannot be entirely excluded.

1.2.4.2 MOG as a potential regulator of complement component C1q

Several studies conducted in the late 1990s implied a connection between myelin and the complement system (reviewed in [36]). Notably, Johns *et al.* reported MOG to bind C1q in a calcium-dependent manner and thereby to inhibit the classical complement pathway [91]. Frankly the implications for MS are intriguing. Complement deposition is a hallmark of MS pathology, triggering demyelination and affecting adaptive immunity [99], and it has been reported that complement component C3 primes microglia [100]. Convesely, complement fulfills a plethora of non-inflammatory housekeeping functions, e.g. the clearance of apoptotic cell debris, pruning of synapses in the CNS [101, 102] or regulation on Wnt signaling [103]. Accordingly, the reported interaction with MOG and negative regulation of C1q [91] potentially has considerable implications for the CNS and MS.

1.2.4.3 Excursion: The discontinued MOG and C1q project

Based on the above, I was naturally intrigued by the potential role of MOG and regulating C1q in the context of MS. To recapitulate the original publication [91] made a few major claims:

1. MOG binds to C1q
2. The binding of MOG and C1q is possibly Ca^{2+}-dependent, (addition of EDTA decreased binding and addition of Ca^{2+} increased it)
3. MOG inhibits the classical complement pathway (demonstrated in a complement-dependent lysis assay of red blood cells)

I initiated an entire project trying to reproduce the claim that MOG binds to C1q, possibly in a Ca^{2+}-dependent manner. In continuation it would be a goal to further experimentally confirm this and to investigate the specific nature and functional consequences. To cut a long story short, despite suggestive initial data, the experiments did not support a specific, nor Ca^{2+}-dependent interaction of MOG and C1q, nor did pure MOG appear to regulate the classical pathway *in vitro* (data summarized in Figure 4). Instead the data raises question about the validity of the above claims.

Initially, investigating the calcium dependence looked somewhat promising. The MOG_{55-73} sequence displayed some partial matching to the canonical EF-hand motif for Ca^{2+} binding (Figure 4A), but there were clear deviations. A generated 3D model localized the putative site to a flexible loop region within MOG (Figure 4B) with possible implications on conformational changes. Importantly, the site localized closely to a ExRxR motif which was proposed in the original publication as the possible interaction site with C1q [91], but this site is localized at the base of the protein facing the cell membrane. The fact that none of the structural publications of MOG detected or implied Ca^{2+} binding also inspires healthy skepticism [60, 90]. Regardless, experimental ELISAs did actually confirm binding of MOG to C1q (Figure 4D), although the signal appears to be more indicative of the degree of MOG aggregation than specific interaction (cf. Figure 4E). In order to interpret the data one needs to know that production batch 9 contained some MOG multimers, while production batch 10 contained

fewer of these. Additionally, re-purification using size exclusion chromatography was applied to separate the monomeric and dimeric fractions (cf. Figure 4D). Monomeric protein clearly displayed reduced detection compared to aggregated protein.

An additional question in this project was also whether PTMs would sterically interfere with C1q interaction and entail functional consequences. Alternatively, MDA adducts are reported to be recognized by certain complement components [104, 105][36] and to induce interaction with the carrier protein. A major technical problem was that MDA-modifications[37] or nitration[38] also promoted aggregation of MOG *in vitro* due to crosslinking, and thus influenced the ELISA readout. In fact, the binding of monomeric proteins (re-purified by SEC in some cases) was low and almost in the range of background. Addition of extra calcium or omission of calcium and the presence of EDTA did not notably increase or abolish binding as previously claimed [91]. Lastly, a functional complement lysis assay on human platelets did not show any functional consequence of MOG being present (Figure 4F). Taken together, this mostly negative data did not encourage continuation of the project. Corresponding to the above claims, I have listed some conclusions and open question of my own:

1. The major determinant of ELISA reactivity appeared to be the presence of MOG aggregates. Is the binding in any way specific, or does C1q merely bind the aggregated and misfolded protein? If so, how was the quality of the MOG used in the original publication?
2. There is no evidence for Ca^{2+} dependence. Did the original publication neglect artifacts induced by precipitation of insoluble $CaHPO_4$, a common mistake when using calcium in combination with PBS-based buffers? [39]
3. Is there any specific function associated with the MOG_{54-73} region?

Concerning this project there were not only technical difficulties and artifacts I became aware of but the overall construct of the project appeared artificial and questionable upon reflection. I decided anyhow to include the data in form of this excursion for two reasons: Firstly I wanted to challenge the claim that MOG actually specifically interacts with C1q. Secondly I intend to highlight that sustainable science requires awareness and thoroughness to allow the recognition of flaws and artifacts, as well as the necessity to report and discuss negative data.

1.2.4.4 MOG as CNS-specific receptor for Nerve growth factor

A more recent study has now revealed that MOG has a hitherto unknown function, namely to bind and sequester Nerve growth factor (NGF) [92]. This function fits to the CNS-specific expression of MOG. In particular, the thorough study by von Büdingen *et al.* [92] demonstrates MOG binding NGF (K_D = 581 pM) and aberrant sprouting of $TrkA^+$ nerve fibers in $MOG^{-/-}$

[36] Potentially MDA adducts are recognized by C1q (Anna Blom, Lund University, personal communication); preliminary experiments.
[37] Possibly targeting K_{55} and/or K_{73}
[38] Possibly affecting Y_{65} (we now know from Paper 3 that Y_{65} is poorly nitrated)
[39] $CaHPO_4$ precipitates easily in solution when Ca^{2+} is added to PBS and may co-precipitate protein; the original publication added up to 5mM Ca^{2+}, standard calcium-containing PBS formulations contain about 0.9mM.

mice. TrkA is an established neuronal NGF-binding receptor tyrosine kinase and is structurally related to MOG, yet more distantly compared to the B7 family. Notably, compared to TrkA MOG features a more extensive loop between the C'' and D strands (residues 61-81), cf. Figure 3, which corresponds to the region studied in connection to C1q.

1.2.4.5 MOG and interaction with DC-SIGN

MOG has been demonstrated to interact with the via C-type lectin DC-SIGN via fucosylated N-glycans on MOG [106]. Together with TLR4 activation this interaction decreased T cell proliferation via the secretion of IL-10 [106]. Conversely, the absence of myelin fucosylation promoted Th17 subsets [106]. These observations imply a tolerogenic role for MOG depending on its glycosylation. Interestingly, dysregulated N-glycosylation has been suggested as a pathogenic denominator in MS [14]. Importantly, there are notable differences in expression distribution between human DC-SIGN (associated to DCs) and its rodent variant DC-SIGNR1 (associated among others to marginal zone macrophages). The tolerogenic signature of DC-SIGN-expressing APCs in the context of glycosylation has been proposed as a therapeutic approach in autoimmunity [106].

1.2.5 MOG IN NARCOLEPSY

A mutation in the MOG gene has been associated with inheritable narcolepsy [107]. This C398G mutation results in a non-synonymous S104C[40] substitution in the MOG protein. The affected residue is located at the very tip of the MOG IgV domain just prior to the final β-sheet, a region known to interact with the 8-18C5 antibody [60]. As opposed to MS, however, there is no evidence for autoimmunity directed towards MOG in the affected narcolepsy patients [107]. Most likely the additional cysteine, as a result of the mutation, interferes in the proper structural folding of the IgV domain and produces non-functional protein. Nonetheless, the role of MOG in the context of narcolepsy remains elusive.

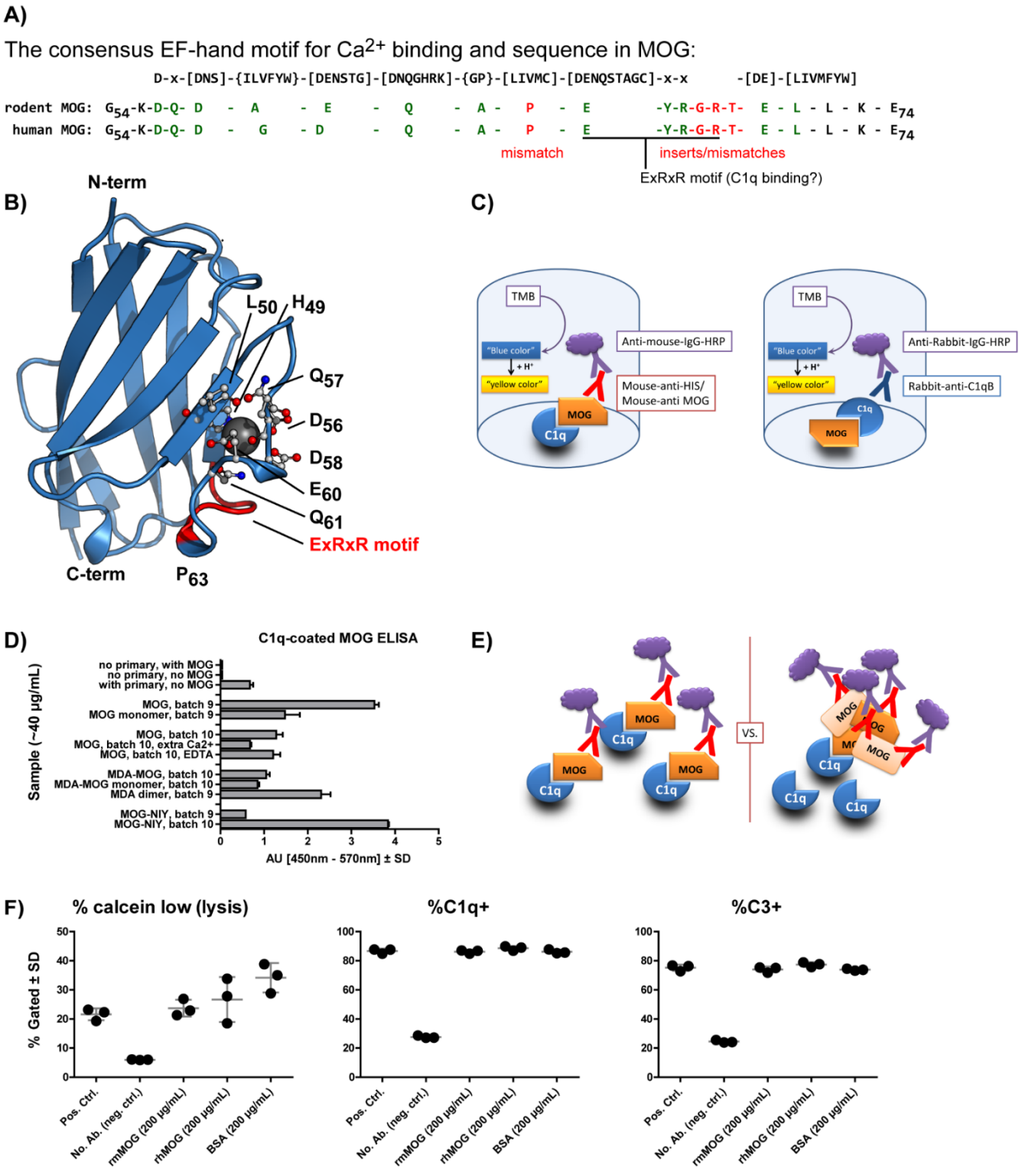
Interest in autoimmune narcolepsy spiked after the link between increased cases and the Pandemrix™ vaccine[41] was established, along with the strong association with HLA-DQB1*0602. However, later studies attributed autoimmune narcolepsy to molecular mimicry and cross-reactive immune responses between influenza nucleoprotein and human hypocretin/orexin (reviewed in [108]), neuropeptides with established roles in regulating wakefulness and food intake. Interestingly, the familial narcolepsy associated with the MOG mutation is also associated with obesity in roughly 50% of the cases [107], but it is neither clear whether this is actually attributable to the mutation nor how it speculatively would be connected mechanistically.

---

[40] The original publication uses the numbering according to the full length precursor sequence, including the signaling peptide (29 residues). S133C corresponds to S104C in the processed version.

[41] H1N1 flu vaccination during 2009/2010 in Europe

Figure 4: The discontinued MOG and C1q project



A) A comparison of the MOG sequences to the canonical EF-hand motif for Ca^{2+} binding according to PROSITE (http://prosite.expasy.org/PS00018). Note that square brackets indicate allowed (required) choices while curly brackets indicate disallowed choices, 'x' can represent any amino acid. The MOG sequences indicated in green match the canonical motif. However, the sequences fail to meet the criteria with P_{63} being a mismatch and $G_{67}R_{68}T_{69}$ being additional inserts/mismatches. Notably, this region overlaps with the ExRxR motif proposed to resemble to the C1q binding motif in antibodies, as indicated(Johns *et al.*, 1997)

B) Based on the crystal structure of mouse MOG [PDB-ID: 1PY9] a hypothetical calcium ion was modeled into the center of potentially chelating amino acids (Glutamic acid, E or aspartic acid, D). Larger structural rearrangements as a result of conformational change were not taken into account. The $E_{64}xRxR_{68}$ sequence is colored red1. The proximal amino acids to the hypothetical calcium ion (grey, 1.85Å radius) are displayed as sticks with oxygens highlighted in red. Modeling the calcium in this position is highly suggestive of D_{56}, D_{58} and E_{60} potentially chelating the ion, however, this would theoretically require the participation of the backbone oxygen of L_{50} as well as the oxygens of Q_{57} and Q_{61} in the chelation and most likely a change in conformation.

C) Schematics for the ELISA setups of C1q-coatead MOG ELISA (left), or the inverted setup with MOG-coated C1q ELISA (right).

D) C1q-coated MOG ELISA data from selected experiments. The controls indicate that there is some background with the antibody, which is possibly bound by C1q. Samples from production batch 9 indicate that there is interaction between MOG and C1q but that the detection is influenced heavily by the amount of non-monomeric, potentially misfolded, forms of MOG. Additional samples from batch 10 reveal not only batch variation (production batch 10 being purer and more monomeric), but also that increasing the calcium concentration or eliminating it in the presence of EDTA does not increase or eliminate the binding, respectively. MDA modification of MOG appeared to increase binding only due the promotion of multimer formation (crosslinking). Nitration also displayed ambiguous effects, also most likely related to the degree of multimer formation. The overall take-home messages from this data are that although C1q possibly binds MOG, or rather aggregates thereof, the interaction cannot be reliably assayed using this setup, and calcium does not appear to make any difference.

E) Concept of MOG multimers negatively affecting the ELISA readout.

F) Experimental data testing the ability of MOG to inhibit the classical complement pathway. Human platelets were prepared by labeling with calcein dye, then incubated in the presence of various proteins and antibodies directed to the platelets and serum. The platelets were subsequently stained and prepared for analysis by FACS. The FACS analysis demonstrated calcein release from lysing platelets in all samples with antibody (left), but the presence of highly pure monomeric MOG or BSA control did not prevent lysis. Similarly the presence of MOG did not prevent the platelets from being coated with C1q or C3.

1.2.6 MOG AS AN AUTOANTIGEN

In the majority of MS-related studies MOG is a tool to induce EAE rather than a subject of research. The latter sadly factors into the reality that comparatively little is known about the physiological functions of MOG, apart from what has been suggested by the individual studies discussed above.

1.2.6.1 MOG as an established MS autoantigen

Quickly after its discovery the research focus shifted towards the role of MOG as an autoantigen in MS and translated as a means to induce EAE (reviewed in [109]). It was quickly established that immunization with MOG protein induced EAE [109]. Shortly after, the immunodominant peptide in C57BL/6 mice was mapped to MOG_{35-55} and the peptide EAE model was established [110]. Subsequently, the presentation and recognition of the MOG_{38-51} core epitope were fine-mapped in functional experiments [111, 112] and it was established that MOG_{35-55} is presented by H2-IA^b, with W_{44} being the major TCR contact [113].

MOG knockout mice are immune to MOG-induced EAE and display milder EAE symptoms in myelin-induced EAE [98]. This implies that although MOG is a minor component of myelin it is a major autoantigen in CNS demyelination.

In the context of the MOG gene structure I discussed the splicing of human MOG [84] and the potential implications for autoimmunity [86]. Notably, the extracellular domain of MOG is not changed by splicing, but a recent study in mice suggested an additional encephalitogenic epitope of MOG residing in the transmembrane region (residues 119–132) [114].

1.2.6.2 MOG epitopes

A few of the major MOG epitopes in rodents and humans are depicted in Figure 3 (pg. 14). In rodent models the dominant epitopes are well characterized, e.g. MOG_{35-55} for mice with the $H2^b$ haplotype (e.g. C57BL/6) or MOG_{79-98} for $H2^q$ mice (DBA1) [115]. Human MOG epitopes are unsurprisingly more heterogeneous and cover most of the MOG IGV domain [116, 117]. Structural evidence for the binding of specific MOG, or myelin epitopes in general, to any of the established risk HLAs is still lacking in MS research. However, the developments in the production of MHC tetramers as well as high throughput analytical methods may open new avenues.

1.2.6.3 MOG antibodies

The location of MOG in the outermost layer of myelin (cf. Figure 2, pg. 4) has always been the motivation to study MOG in the context of autoantibodies. In human MS the situation concerning MOG-specific antibodies is considerably complex, partly due to controversies in studies trying to establish a role for MOG autoantibodies in MS (reviewed in [37]). Notably,

the patient heterogeneity in MS could potentially be stratified according to antibody serology profiles.

In rodents the antibody responses are reported to mainly recognize folded MOG [118]. This is consistent with reports suggesting that MOG antibodies are generally conformational [41], and the fact that the MOG-defining 8-18C5 antibody is also conformational [36]. The crystal structure of rat MOG in complex with 8-18C5 has been resolved and this revealed that the antibody recognizes stretches from apical regions in MOG [60].

Several studies in mice imply that MOG antibodies are expendable for CNS demyelination, but in certain cases they can exacerbate demyelination. Conversely, not all MOG antibodies are capable of driving pathology [37], which may be connected to recognition of conformational or linear epitopes [119]. Importantly, the APC function, not the antibody production, of MOG-specific B cells is now identified as a critical contributor to MS pathogenesis [32].

The fact that there are several protective class I alleles associated with MS [51] suggest viral infection, or clearance thereof, as a potential factor in MS pathogenesis. Relating to that, MOG has been suggested to be an adhesion molecule for *Rubella* virus [120]. Notably, this further relates to intrathecal production of anti-*rubella* IgG in MS patients [30]. The latter may indicate putative bystander activation related to the virus. The WHO recommends to make *Rubella* vaccinations routine to prevent outbreaks and birth defects associated with the virus [121]. Sweden vaccinates all children using combination vaccines, but women may receive booster vaccinations in case antibody titers are tested low.[42] However, to my knowledge there is no epidemiological evidence that would support a connection of R*ubella* vaccinations to MS in these women, although this question could easily be addressed using the registries.

1.2.6.4 Propagation of demyelination in the CNS may require a secondary priming with endogenous MOG

The original priming with MOG in adjuvant appears not to suffice for EAE induction alone. The literature provides several lines of evidence that the propagation of demyelination in the CNS may require a secondary priming with endogenous MOG. Initial evidence for this comes from the observation that EAE induced by either rat or human MOG is driven by different mechanisms [122]. This is attributed to the S42P substitution in human MOG, implying that the EAE induced with human MOG_{35-55} peptide relies on the induction of a secondary response to the rodent version. More recently it was demonstrated that the EAE driven by human MOG is B cell-dependent [32]. Arguably, immunization with human peptide is highly artificial. However, the antigen processing machinery requires the IFNγ-inducible lysosomal thiol

[42] *Rubella* infections in Sweden have since been practically eradicated, apart from rare imported cases or outbreaks associated to anti-vaccine communities in Järna (2012); source: Folkhälsomyndigheten (Swedish health authority).

reductase (GILT) in order to release to MOG_{35-55} epitope [119]. GILT is the product of the MS risk gene IFI30 [54], and is required to reduce disulfide bonds in order for antigens to be processed. $GILT^{-/-}$ mice are susceptible to EAE induced with rat MOG, but react to MOG epitopes from non-globular intracellular regions mentioned above. Conversely, $GILT^{-/-}$ mice also display an abundance of plasma cells and pathogenic MOG antibodies [119], which is not surprising for mice that have a deficiency in unfolding antigen into linear epitopes. Importantly, and surprisingly, $GILT^{-/-}$ mice are essentially resistant to MOG_{35-55} peptide EAE [119], although one might expect the opposite. In other words, MOG_{35-55}-primed $GILT^{-/-}$ mice have an ongoing T cell response that fails to propagate in the CNS because that requires local antigen presentation of the MOG_{35-55} epitope.

Taken together, these data collectively imply that A) the initial T cell response to MOG_{35-55} only induces minimal CNS demyelination and relies on propagation in the CNS, and B) processing events of endogenous MOG are required to enable this propagation. In conclusion, re-stimulation of infiltrating T cells by local APC in the CNS is already recognized as an important factor [1].

1.2.7 CONCLUDING REFLECTIONS ON MOG

In summary, it appears that MOG has evolved in a very unique fashion to fulfill a range of functions that contribute to the CNS microenvironment. Together these may impact the developing or inflamed CNS in various ways, and there is more to be learnt about MOG itself and its role in CNS homeostasis. The establishment of MOG as an autoantigen in MS and its subsequent use as a tool to evoke EAE may have diverted efforts to elucidate its function. However, the insights gained from years of research using the EAE model have greatly deepened our understanding of MS.

1.3 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Pre-clinical research that aims to dissect disease mechanisms prior to clinical presentation relies on reproducible models that mimic the pathology and relevant aspects of the human disease. For MS research, this is the EAE model, Experimental Autoimmune[43] Encephalomyelitis.

1.3.1 THE HISTORY OF EAE

The concept of experimentally-induced CNS inflammation originated in the mid 1920s (reviewed in [123]). Cornerstone observations relate to CNS inflammation induced by exposure to CNS extracts, most notably non-human primates during the elucidation of encephalitogenic complications with a rabies vaccine contaminated with myelin components (reviewed in [123]). Later, the use of Freund's adjuvant[44] enabled researchers to significantly boost immune responses and thus to induce EAE with a standardized single immunization (reviewed in [123]). Since then EAE has been induced in numerous species including mice, rats, rabbits and non-human primates in order to study immune mechanisms related to CNS inflammation.

1.3.2 THE EAE MODEL

1.3.2.1 Active EAE

In essence the active EAE model boils down to immunizing animals with CNS antigen emulsified in adjuvant. The detailed aspects of this are reviewed in the literature [27, 124].

There obviously are many factors to tweak in the process of EAE induction. First, different animal species and strains react differently to the same immunization protocol. These differences reach as far as strains of the same background being either resistant or susceptible to EAE depending on the MHC haplotype [115, 125] or on individual non-MHC gene loci [126].

Secondly, the choice of antigen is critical. Pioneer studies used whole myelin extracts as antigen, but those inherently have significant variability in their composition. Recombinant or purified proteins and synthetic peptides thereof, i.e. defined antigens, are therefore currently the primary choices for EAE induction. Notably, the combination of a specific strain and antigen evokes variable disease courses that can be relapsing-remitting, chronic or progressive [27]. The disease course is furthermore influenced by the antigen dose and strength of the adjuvant, higher doses and stronger priming promoting EAE severity. EAE typically presents

[43] Earlier studies refer to EAE as Experimental *Allergic* Encephalomyelitis, owning to the fact that the autoimmune aspect was unclear at the time.
[44] Incomplete Freund's Adjuvant (IFA): 85% (v/v) paraffin oil, 15% (v/v) mannide monooleate; Complete Freund's adjuvant (CFA): IFA + heat-killed *mycobacterium tuberculosis* (500-2000 µg/mL in final emulsion).

with a progressively ascending paralysis of tail and limbs and is monitored using an ordinal scale.

1.3.2.2 Pertussis toxin

In certain strains of mice the successful induction of EAE symptoms requires the intraperitoneal co-administration of ~200 ng *pertussis* toxin (PTx) on days 0 and 2. The requirement for Ptx administration relates to genetic susceptibility: for instance the C57BL/6 strain requires PTx, whereas the DBA1 strain does not [115][45]. The precise effects of PTx are incompletely understood but include genotype-dependent bystander activation [127], skewing of $CD4^+$ T cells towards the encephalitogenic Th17 phenotype [128] and transient breakdown of BBB integrity with structural aberrations of organelles in microvascular endothelial cells [129]. Conversely, repetitive administration of PTx prior to immunization was reported to promote regulatory T cells and to counteract the development of EAE [130]. Furthermore, PTx-induced BBB permeability is transient (~24h) [129], whereas EAE symptoms do not develop prior to ~10 days after immunization[46].

1.3.2.3 Passive EAE

In contrast to active EAE, passive EAE does not involve immunizing the animal with antigen emulsion but rather involves T cell transfer [124, 131]. The passive EAE model itself was originally developed to demonstrate T cells being the pivotal mediators of disease in EAE [132]. For passive EAE the transferred T cells derive from actively immunized donor animals and are typically propagated *in vitro* by rounds of stimulation with the particular antigen and IL-2 [124, 131]. Furthermore, the Th subset of $CD4^+$ T cells can be skewed by addition of the required cytokines such as IL-12 for Th1, or IL-6 and IL-23 for the Th17 subset [65, 131].

A recent study suggests that transferred T cells acquire a migratory phenotype after initially homing to the lungs [23]. These licensed T cells transgress into the CNS and induce demyelination, evoking EAE symptoms that are often monophasic [124, 131]. Importantly, the Th subtype influences the location of inflammation, Th17 cells driving inflammation in the brain parenchyma and evoking 'atypical' EAE symptoms [20, 133, 134]. 'Atypical' EAE symptoms are characterized by unsteady gait, ataxia, and disturbances in axial and rotary orientation, often lacking the ascending paralysis that is typical for classical EAE.

[45] DBA1 mice also require less CFA (~50-100 µg) and lower protein doses (<25 µg).
[46] i.e. 8 days after the last PTx administration.

1.3.3 2D2 MICE

A extremely useful resource in assessing MOG-specific proliferation are 2D2 mice [135] which were developed on a C57BL/6 background and are transgenic for TCR chains recognizing MOG_{35-55} (Vα3.2 and Vβ11), derived from a MOG_{35-55} -reactive T cell clone [135]. Using functional studies the fine reactivity was later mapped to the core MOG_{38-51} epitope [111] which is presented by H2-IA^b [113]. 2D2 mice are very useful in experimental research because they are a source of T cells with pre-defined reactivity towards MOG. Importantly, 2D2 mice allow bypassing of immunization of wild type mice and generating polyclonal T cell lines, although this approach may still be necessary depending on the research question.

Notably, 2D2 mice are not the only MOG-specific TCR transgenic mice. Mendel *et al.*, developed the so-called 13A strain (TCR Vα2/Vβ4) using essentially the same approach [136]. However, the A13 strain is resistant to active EAE, and T cell transfer of Th1 activated A13 T cells does not induce EAE in wild type mice [136]. Furthermore, A13 T cells secrete IL-4 and undergo apoptosis when stimulated with MOG_{35-55} and IL-12 [136]. Taken together, it appears remarkable that these strains exhibit such notably divergent phenotypes despite the only apparent difference between them being the transgenic TCRs.

1.3.4 OTHER MODELS OF CNS AUTOIMMUNITY AND DEMYELINATION

There is a range of uncommonly used spontaneous models for CNS infiltration, most of which rely on removal of regulatory T cells (e.g. by thymectomy) or ectopic expression of cytokines (reviewed in [137]). Other models of CNS demyelination include Japanese macaques [138] and Theiler's virus induced demyelination [124]. Furthermore, there are additional chemical models [27] and genetically engineered mice [139, 140] that address demyelination and CNS infiltration [141].

While there are several transgenic mice with TCRs specific for particular myelin antigens [137], the incidence of spontaneous EAE is mostly low. For instance, less than 4% of the widely used 2D2 mice develop spontaneous EAE symptoms [135] although 30% present with optic neuritis. The fact that 2D2 mice do not develop EAE despite having transgenic T cells is enigmatic with respect to how difficult it can actually be to break tolerance. More recently, however, it has been demonstrated that transgenic mice with both 2D2 TCR and MOG-specific B cells have up to 78% spontaneous EAE incidence [32, 142][47].

A recent study reports a novel model of immune-mediated CNS demyelination [143] in which administration of tamoxifen to transgenic mice induces oligodendrocyte death. Treated mice develop a late-onset disease characterized by motoric disturbances, the presence of MOG-

[47] 0% in either MOG-specific B cells or T cells alone; 55% with both 2D2 TCR and MOG-specific B cells deficient in antibody secretion; 78% with both 2D2 and MOG-specific B cells able to secrete antibodies.

reactive T cells, secondary demyelination and CNS infiltration [143], but appear to lack the limb paralysis typical for EAE. This phenotype thus possibly resembles the disability and chronicity of early MS better than does the EAE model. Whether oligodendrocyte death elicits CNS autoimmunity has been controversial in earlier studies which focused on earlier time points [139, 140].

1.3.5 TRANSLATIONAL LIMITS OF THE EAE MODEL

The EAE model is widely used in MS research and has been instrumental in identifying several mechanisms relevant to CNS autoimmunity [27]. Especially the use of various knockout mice, notably cell type-restricted knockouts, offers immense potential into addressing the relevance of particular targets [19, 54]. Among many others these models have highlighted the importance of T cell-derived GM-CSF [25] for inflammatory $CCR2^+$ myeloid cells [24] for mediating demyelination as opposed to IFNγ [144] or IL-12p35 [145]. The EAE model also offers options to address environmental factors such as the microbiome [146] and diet [147, 148].

Although the EAE model is instrumental in understanding CNS inflammation, it fails to fully mimic the complexity of human MS. Compared to MS as a heterogeneous syndrome EAE is inherently a controlled, yet very harsh and highly artificial model [1]. Active EAE has an inherent $CD4^+$ T cell bias owing to the use of adjuvants [1, 27]. Furthermore, the location of lesions and the chronicity aspect of MS are poorly reflected in the EAE model [1, 141]. Monitoring demyelination vs. axonal degeneration can easily be inaccurate [149] and there is especially a lack of models resembling mechanisms related to progressive MS. The EAE model itself is very susceptible to treatment interventions, environmental factors and technical variation [146, 149]. Lastly, mice are not humans and there are notable differences in the respective immune responses. These differences impinge on both mechanisms and treatments identified using EAE. For instance, even though TNF has been implicated to drive EAE, anti-TNF treatment, although successfully used in the therapy of RA, turned out to be detrimental in clinical trials against human MS [150].

Taken together, these and other limitations highlight the importance of critical assessments when drawing conclusions about clinical MS based on observations in EAE. Unless experiments are performed with good preparation and execution, randomization and blinding, they bear a significant risk for technical or subjective bias. Furthermore, awareness of potential limitations is a caveat for sustainable research performance.

1.4 POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications (PTMs) are discussed in the introductions of the respective studies (I-III) and I consider it redundant to discuss these in more detail in the frame of the thesis. Instead I decided to focus the frame discussion more strongly on malondialdehyde (MDA) as it relates most to my research (especially Study I) and allows me to discuss details in a way that may not be possible in standard publications. Here I will very briefly discuss some of the concepts of PTMs, but otherwise refer to existing reviews [151, 152].

1.4.1 POST-TRANSLATIONAL MODIFICATIONS IN HOMEOSTASIS

PTMs are covalent chemical modifications of amino acids that occur in proteins after synthesis [153, 154] and they vastly increase the variety of amino acids in proteins. The majority of PTMs occurs naturally and is mediated specifically by enzymes in order to (reversibly) regulate cellular signaling or metabolic processes. The best example is phosphorylation in signaling cascades being regulated by kinases and phosphatases.

Conversely, the majority of non-enzymatic modifications are associated to disturbance to cellular homeostasis, but are normally taken care of. There are many examples for these different modification, but I refer to Study II for additional details.

Importantly, many PTMs occur naturally and constantly in the body. My favorite example is the conversion of asparagine to iso-aspartate [153, 155]: during the conversion the protein backbone is linked instead to the side chain and introduces irregularities. Repair of iso-aspartate is repaired by the enzyme Protein L-isoaspartyl methyltransferase (PIMT). Deficiencies in PIMT are embryonically lethal in knockout mice that display accumulation of iso-aspartate residues, especially in the brain, and severe seizures [156, 157]. This examples highlights both how common PTM are and how relevant their repair is [158, 159].

1.4.2 MECHANISTIC LINKS OF PTMS IN AUTOIMMUNITY

1.4.2.1 A brief outline of the theory of PTMs in autoimmunity

Mechanisms of PTMs in autoimmunity have been previously reviewed [151, 152]. Here, I will simply go over them to introduce some terminology. The major underlying concept of PTMs in autoimmunity is that they impact protein recognition or processing by introducing novel epitopes, called *neoepitopes*. If the epitope contains a PTM, it is called an *altered peptide ligand (APL)*. Conversely, if the epitope is released by altered protein processing events it is referred to as *cryptic epitope*. The theory of PTMs in autoimmunity assumes that *neoepitopes* are absent during *negative selection* of potentially *autoreactive* T cells in the thymus. These autoreactive T cells, having escaped *central tolerance* mechanisms, are the basis for adaptive autoimmunity.

Consider that we are following an antigen from the point it becomes modified until it evokes an immune response. The modified antigen might resemble a protein the immune system recognizes as foreign ‘non-self’ or a pathogen due to similarities in topology or sequence. This

is referred to as *molecular mimicry* [160] and implies *cross-reactivity* of antibodies or T cells, with possible *bystander activation* during an ongoing infection.

Modification of the antigen possibly entails *innate recognition* by *scavenger receptors* [161] and enhanced uptake into *antigen presenting cells (APCs)*. This active mode of receptor-mediated phagocytosis, as opposed to more passive modes of uptake, is highly effective and possibly activates cellular signaling pathways and thereby the APC. Activated APCs possibly contribute to the inflammatory microenvironment by their secretion of cytokines and expression of surface molecules. The critical steps for induction of adaptive autoimmunity are *antigen processing* and *antigen presentation*. A mentioned above the PTM may interfere with processing events, e.g. by saving a cryptic epitope from *destructive processing* [162], or changing the *processing cascade* entirely [163]. Alternatively, the epitope can contain the PTM and be an APL. This possibly enables *epitope spreading* to new determinants of the same protein, or a new target. The generated epitopes compete for binding to *major histocompatibility complexes (MHC)*, the epitopes with highest affinity to a given MHC molecule being more likely to be presented [52].[48] Lastly, all above events culminate in the recognition of a presented antigen by an autoreactive T cell and its activation. This brief recap may be slightly over-simplistic and fails to fully elucidate the complexity of the theory of PTMs in autoimmunity or explain examples in detail, but the purpose is to give an impression.

1.4.3 PTMs IN MS

The best-established involvement of PTMs in autoimmunity is citrullination in the Rheumatoid Arthritis (RA) field [52, 164], possibly also the related carbamylation [165, 166]. Conversely, in MS a potential involvement of PTMs is currently not conclusively established. Existing studies have addressed the immunogenicity of citrullinated MBP, which is rather complex as MBP itself in naturally citrullinated multiple times [167, 168]. There is furthermore evidence of epitope spreading to citrullinated antigens in EAE [169]. Another compelling example is that the $MBP_{Ac1\text{-}11}$ epitope requires N-terminal acetylation in order to be presented by I-A^u and induce EAE [170].

Furthermore, the contribution of oxidative stress and particularly lipid peroxidation were implied in the pathogenesis of MS [171-173]. Lipid peroxidation in MS [172, 173] and potential PTMS associated to oligodendrocyte death [174] therefore warrant researching the mechanisms in relation to MS pathogenesis. This has been a major factor behind Studies I-III and is discussed separately in their respective introductions. In the next chapter I will focus on introducing the background to lipid peroxidation and MDA modification.

[48] This is well established for citrullinated APLs and their presentation by risk haplotypes in Rheumatoid Arthritis.

1.5 MALONDIALDEHYDE

1.5.1 LIPID PEROXIDATION

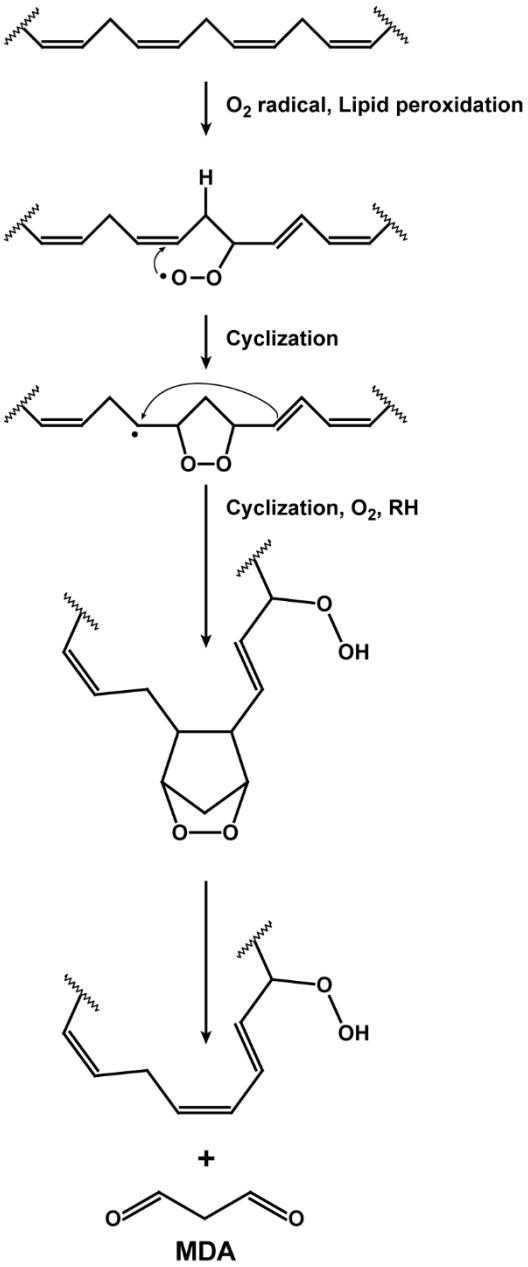
1.5.1.1 Lipid peroxidation gives rise to reactive aldehydes

The major cause for the generation of MDA *in vivo* is lipid peroxidation [175, 176], in particular of poly-unsaturated fatty acids such as membrane arachidonic acid (AA) and docosahexaenoic acid (reviewed in [175]). Lipid peroxidation can be mediated in a comparatively controlled manner by enzymes with peroxidase activity for various synthetic or metabolic pathways (reviewed in [175]). Conversely, uncontrolled lipid peroxidation is considered a pathophysiological process driven by ROS [175-177] or RNS [178]. Apart from exogenous sources, ROS derived from enzymes including iNOS and NOX may contribute to this process [179].

Lipid peroxidation as a separate subject field is very complex and reviewed elsewhere [177]. Importantly, several pathways have been described, though which downstream processes initiated by lipid peroxidation yield highly reactive aldehyde compounds including 4HNE [176, 180] and MDA [176, 177, 181, 182]. Reaction products of MDA and 4HNE with biomolecules belong to a category collectively referred to as advanced lipoxidation end products (ALEs) [183]. Both MDA and 4HNE have been studied extensively as biomarkers of oxidative stress [184] and are implicated in the pathology of various diseases.

A second major source of MDA is its generation as a byproduct in the synthesis pathway of thromboxane A2 (TXA2), although this is technically enzymatic lipid peroxidation. In the synthesis process MDA can be released from prostaglandin H2 which is in turn generated from arachidonic acid via cyclooxygenases (COX1/2) [185]. A schematic for this particular source is described in [181], but mechanistically the decomposition step releasing MDA corresponds to that depicted in Figure 5 (pg. 32) [176, 177].

Figure 5: Schematic MDA generation by lipid peroxidation



This simplified schematic depicts the generation of MDA by lipid peroxidation.

The initiating step is lipid peroxidation, i.e. the attachment of peroxide to the poly unsaturated fatty acid (PUFA) chain of a lipid. This lipid peroxidation can be initiated by ROS, or enzymes with peroxidase activity.

The peroxidized lipid subsequently undergoes two steps of internal cyclization, first internally forming an endoperoxide, secondly forming a 5 carbon ring. Rearrangements within the lipid radical further entails additional peroxidation until scavenging of a hydrogen atom occurs.

The resulting bicyclic endoperoxide can be a precursor for isoprostanes, thromboxanes and leukotrienes in enzymatic synthesis pathways, but readily decomposes to release MDA.

Adapted from (Nam, 2011).

1.5.1.2 Antioxidant scavenging counteracting lipid peroxidation

Lipid peroxidation is driven by free radicals. Accordingly, antioxidant defense mechanisms are highly relevant to limit the occurrence and extend of lipid peroxidation [186]. The most notable radical-scavenging antioxidants in relation to lipid peroxidation are α-Tocopherol (vitamin E) and linoleate [187], but other known antioxidants such as thioredoxin or glutathione are possibly also relevant [184, 188]. Importantly, radical-scavenging activity is essential in limiting the potentially detrimental consequences of ongoing lipid peroxidation [182, 186].

In relation to antioxidants it should be noted that factors that generally deplete the antioxidant capacity accordingly increase the risk of pathological processes being initiated by free radicals.

The latter agrees with smoking being a major risk factor for autoimmune diseases in general [56] and the increase of MDA-derived metabolites in urine in smokers [175]. Furthermore, aldehydes in cigarette smoke have been independently reported to react with MDA to form advanced reaction products [189, 190], and I will further discuss these below.

Lastly, although technically not a radical-scavenger, the established COX2 inhibitor aspirin has been demonstrated to reduce both TXA2 and MDA[191], the latter often being used as readout for TXA2 synthesis [192]. This relates to the synthesis of TXA2 being driven by COX1/2 and generating MDA as a byproduct.

1.5.1.3 Other sources of MDA

Urine excretion products of MDA are influenced by thyroid hormones [193], diet [194], physical exercise, and smoking [175]. Apart from lipid peroxidation as the main source it has been suggested that MDA is generated by ionizing irradiation of carbohydrates [195], oxidation of the polyamine spermine [196] and UV radiation [197], in particular UV radiation of the lipid squalene [198]. Interestingly, squalene occurs naturally inside cells, but is also used in adjuvant formulations such as MF59 or AS03 [199]. Squalene by itself, however, does not elicit immune responses [200] and is used as dietary supplement and in cosmetics. Micelle formation is likely the critical step in conveying adjuvant properties of the mixture.

1.5.2 MDA CHEMISTRY

1.5.2.1 Generating MDA in vitro

MDA is highly reactive and is not stable in its pure form. The standard method to generate MDA freshly for laboratory purposes is therefore through acid hydrolysis of 1,1,3,3-tetramethoxypropane [201], which is depicted in Figure 6A (pg. 36). Similar to other aldehydes MDA is suggested to be more stable as its enol form, also called β-hydoxy-Acrolein, especially at low pH [202].

1.5.2.2 Simple MDA reactions with primary amines

MDA is a bifunctional aldehyde and is highly reactive. As aldehydes undergo condensation reactions with primary amines, it is not surprising that the initial reports of MDA adducts reported the Nε amines of lysines to be the main target of MDA [203]. Likewise, MDA also reacts with the primary amine of any amino acid N-terminus [204]. The latter has special relevance for the formation of Nα MDA adducts of phosphatidyl -serine in membrane lipids [205].

There has been some debate about the nature of simple MDA adducts [203]. The reaction of an aldehyde with an amine normally yields a Schiff base, i.e. an imine with a double bond at the nitrogen. However, since MDA is tautomeric it has been suggested that instead the enamine-al variant is the major adduct, featuring the double bond between the MDA carbons and an aldehyde as functional group (cf. Figure 6, pg.36) [203]. Besides lysine, other amino acids, namely arginine, glutamine and histidine, were reported to be modified by MDA [204]. However, the latter remains to be verified, as the chemical nature of the corresponding functional groups does not support modification by MDA.

The imine bond formed between MDA and primary amines is in principle reversible by hydrolysis and is possibly pH dependent [206]. The reversibility has implications for the stability and half-life of simple MDA adducts in carrier proteins potentially acting as reservoirs. The availability of primary amines can be assayed using the TNBS assay [207], whereas detection of MDA adduct is performed using biochemical assays with adduct-reactive antibodies [208] or by Mass Spectrometry [209].

Lysine has a pKa of 10.67 and is expected to be protonated and positively charged at physiologic pH. Conversely, the evidence from various publications suggests that proteins modified by MDA become more net negatively charged (cf. Study I). The chemical reasoning is that both the Schiff base (imine) or alternatively the enamine do not favor the nitrogen being charged. Hence it is reasonable that the nitrogen becomes deprotonated in the process.

1.5.2.3 Generation of advanced MDA adducts: FAAB, MAA and others

The fact that MDA features two reactive aldehyde groups complicates its reaction spectrum significantly. Along with the notion that simple MDA adduct may not be stable it has become apparent that MDA-modified proteins display features that simple MDA adducts do not explain, namely increased spectrophotometric absorbance and the development of color[49].

Accordingly, advanced MDA reaction products were reported in the literature. These include the Malondialdehyde-Acetaldehyde adduct (MAA) [203, 210, 211], the FAAB[50] adduct [210], as well as various crosslinks [203, 211, 212]. Reaction schemes for selected MDA-dependent adducts are depicted in Figure 6 (pg.36).

The most studied advanced MDA adduct is MAA, which is formed by the reaction two units of MDA and a third aldehyde, in this case acetaldehyde, with a primary amine. This reaction forms a dihydropyridine (DHP) ring at the amine that cannot be hydrolyzed. The increased

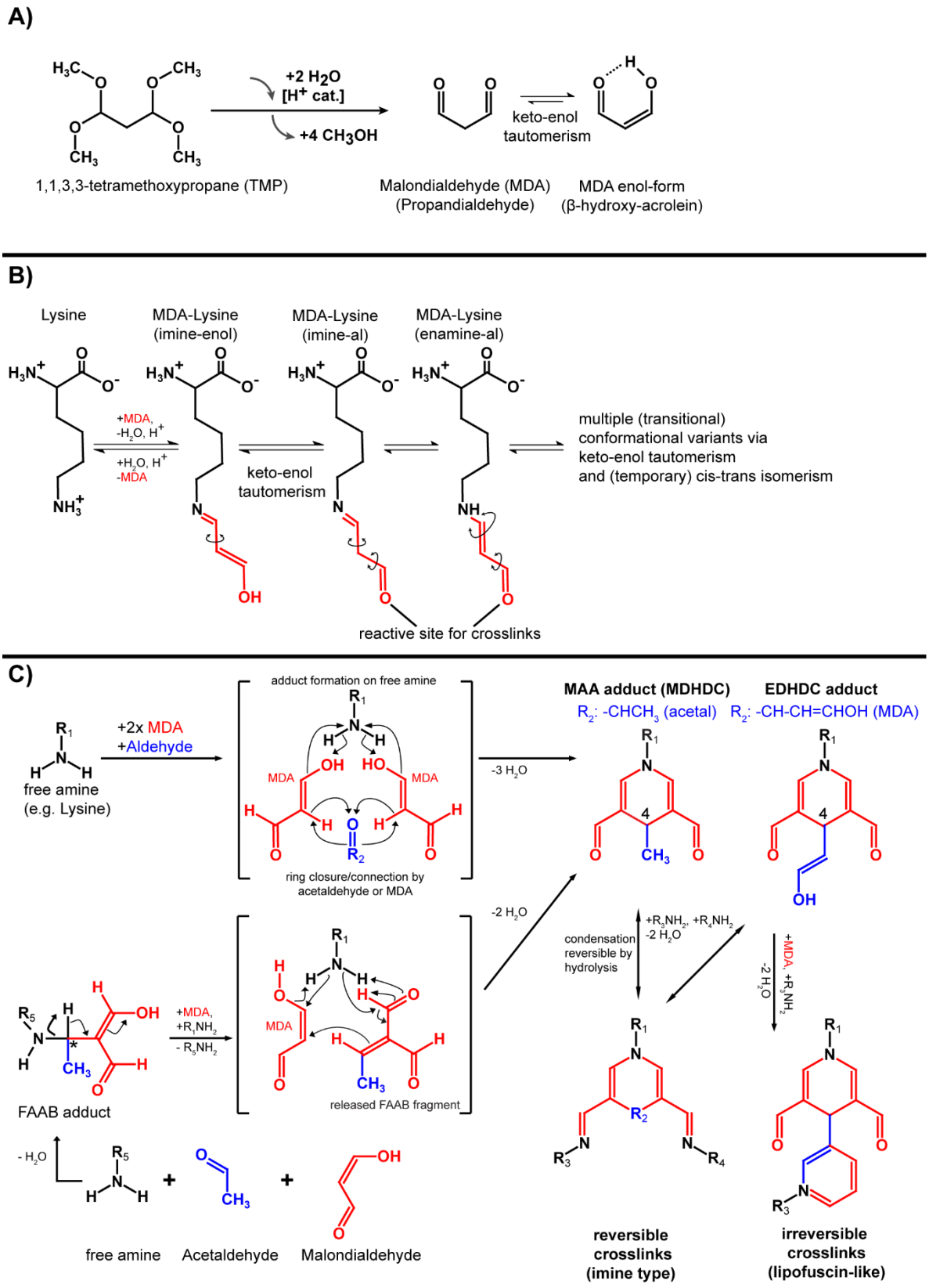
[49] These were among the observation I initially came across when working with MDA. The Nanodrop-based determinations of protein concentration would be unusable and grossly overestimate the yield, due to increased absorbance. Samples would also develop pale yellow colorations.
[50] FAAB: 2-formyl-3-(alkylamino)butanal; This adduct has been reported, but never convincingly demonstrated to be of any significant importance.

stability is relevant as many studies attribute observations to the MAA adduct rather than the MDA simple adduct.[51] The exogenous addition of acetaldehyde to the reaction promotes the formation of MAA adducts [213]. However, the addition of acetaldehyde is not required as MDA itself releases acetaldehyde during decomposition, a step that is rate-limiting [214]. MAA adducts are thus generated *in vitro* even in the absence of the addition of acetaldehyde, but the yield is lower[52] [213]. An alternative MAA reaction mechanism involving conversion of the FAAB adduct has been proposed [215]. Importantly, MAA adducts are reported to form *in vivo* [210] with potential implications of alcohol oxidation to aldehyde.

Alcohol is oxidized *in vivo* by alcohol-dehydrogenases to acetaldehyde, transported via the blood and exhaled via the lungs, as acetaldehyde that is extremely volatile. Historically, acetaldehyde has been used by the police to test drivers for alcohol consumption using exhalation tests, but is modernly detected using spectrophotometry. Interestingly, the reaction mechanism of MAA links alcohol consumption, smoking and the lung microenvironment. The lungs have been demonstrated to be relevant for T cell licensing [23] and either acetaldehyde or aldehydes in cigarette smoke [189, 190] have been demonstrated to react to advanced MDA adducts.

[51] The reasoning behind attributing observations to the MAA adduct often relates to elevated recognition of modified proteins by MDA-reactive antibodies. This could imply both more effective generation, but also prolonges stability.
[52] See also: Supplementary material of Study I

Figure 6: MDA chemistry



A) *In vitro* generation of MDA from 1,1,3,3-tetramethoxypropane by acid hydrolysis. The acid catalyzes the hydrolysis of the methoxy ethers to methanol. Complete hydrolysis yields MDA. MDA is tautomeric with its enol form (aka β-hydroxy-acrolein) suggested to be favored.

B) The reaction of lysine with MDA generates an MDA adduct on the primary amine presumably by forming a Schiff base (imine). However, it is suggested that the final adduct of MDA tautomerizes to an enamine-al. This adduction of MDA to lysine is in principle reversible by hydrolysis and may be pH-dependent. The keto-enol tautomerism is highlighted to illustrate conformational challenges associated to Study II, flexible rotations are indicated by arrows. Notably, the aldehyde in imine-al or enamine-al adducts have the capacity to form crosslinks with other amines by the same reaction.

C) This scheme combines the formation of the MAA adduct by two different mechanisms.
Pathway 1: The upper path illustrates a simplified version of the mechanism proposed by Slatter *et al.* (1998) involving essentially a serial reaction of two units of MDA (red) and an aldehyde (blue) to form the MAA adduct dihydropyridine ring. Note that the ring-closing aldehyde could essentially be any aldehyde, including a third unit of MDA.
Pathway 2: The lower pathway put forward by Tuma *et al.* (2001) instead proposes that the aldehyde reacts first with the amine followed by MDA to form the FAAB adduct. This FAAB adduct is then supposedly released in a reaction with a different amine and another unit of MDA, finally also yielding the MAA adduct. The asterisk indicates a chiral center for the FAAB adduct.
Crosslinks: The carbaldehyde groups of the MAA adduct can condensate with additional primary amines to form crosslinks. Similar to the simple MDA adducts in B, these principally be hydrolyzed and are thus reversible. The advanced reactions of the EDHDC adduct formed by three units of MDA also has the possibility of reacting further with additional crosslinks. However, if the reaction involves a fourth unit of MDA, as indicated, then the crosslink becomes irreversible and forms the lipofuscin-like crosslink adduct reported by Itakura *et al.* (1996). All four adducts on the right half have fluorescent properties.

Square brackets indicate transitional non-existing intermediates.
FAAB: 2-formyl-3-(alkylamino)butanal
MAA: Malondialdehyde-acetaldehyde adduct
MDHDC: 4-methyl-2,6-dihydopyridine-3,5-dicarbaldehyde
EDHDC: 4-ethenol-2,6-dihydopyridine-3,5-dicarbaldehyde

1.5.2.4 Problems: MDA in vitro production, crosslinks, oligomerization, artifacts

Despite being widely used, the *in vitro* production of MDA-modified proteins does not resemble physiological conditions [216], a fact that warrants criticism, in particular concerning:

1) Artificial, uncontrollable reaction conditions and adduct heterogeneity
An issue is the abundance of MDA for *in vitro* preparations being artificially high. One may criticize that the applied concentrations may not resemble those generated during lipid peroxidation. This may not be an issue if the aim was merely to achieve a homogenous modification, but the ensuing problem is the reactivity of MDA. Various potential MDA-derivatives were discussed in the preceding section, and apart from providing acetaldehyde to enforce DHP ring closure and MAA adducts one has relatively poor control over the reaction mechanism as a whole (cf Figure 6, pg.36), and among others MDA inevitably produces crosslinks [203, 211, 212].[53]

2) Incomplete hydrolysis.
Additional issues with MDA relate to incomplete hydrolysis during *in vitro* production and its ability to oligomerize. There is no guarantee that the hydrolysis of TMP as parental compound for MDA is complete, thus giving rise to artificial aldehydes that result in a much wider spectrum of adducts than anticipated.

3) MDA oligomerization.
At the concentrations used *in vitro*, and in the absence of amines, MDA immediately starts to oligomerize [217, 218], and giving rise to a multitude of condensation products and turning gradually from pale yellow to dark red. Pioneer studies on MDA already characterized the divergent properties of the condensation products and warned of the potential pitfalls of studying effects related to those rather than to MDA [219, 220].

4) Insufficient dialysis.
This problem is probably the most relevant one, especially in terms of distinguishing the effects mediated by MDA protein adducts as opposed to those of soluble MDA. Dialysis is the method of choice to clean up the adduction reaction. Two main factors determine the dialysis efficiency: buffer excess and the number of dialysis rounds. Importantly, excess dialysis buffer only determines the relative exchange, e.g. ~1:1000, while the number of rounds increments the efficiency exponentially, i.e. ~$1:1000^{n}$. Importantly, MDA itself has toxic and mutagenic effects at doses of 100 nM or above [205] (discussed below), so the question arises whether certain effects are not attributable to adducted protein, but rather to artifacts due to insufficient dialysis. The concentrations at which the adducted proteins exert their reported pro-

[53] The degree of crosslink formation is correlates to the protein concentration used (unpublished data).

inflammatory effects are often excessively high and correspond to doses that are also toxic to cells [221]. Hence this may imply a titration effect of contaminants. This is especially a risk if MDA-modified protein is prepared 'fresh' and dialysis equilibrium times are cut short. Even one overnight round of dialysis only reduces remaining MDA by only ~1000-fold, depending on the excess buffer. Given that protein is modified at >50 mM, this would leave the free MDA contaminants somewhere below 50 µM and eventually at ~1 µM, assuming a 2 mg/mL protein solution is diluted to 50 µg/mL. The final concentration of contaminants would thus be well above toxic ranges for MDA.

5) Fluorescence. The fact that MDA-derived oligomers and adducts have fluorescent properties interferes with readouts that rely on fluorescence as a readout, most notably FACS. MAA adducts display absorbance around 350-450 nm (peak at 390 nm), which may differ for various MDA oligomers. Importantly, there is detectable emission in FACS channels[54], in particular FL9 and FL10, which bleed into FL1 and FL2 channels that are commonly used for detection. The problem is demonstrated in Figure 7 (pg. 40). Thus for analysis of MDA adducts these channels are best avoided, because there is a significant risk of observing skewed results.

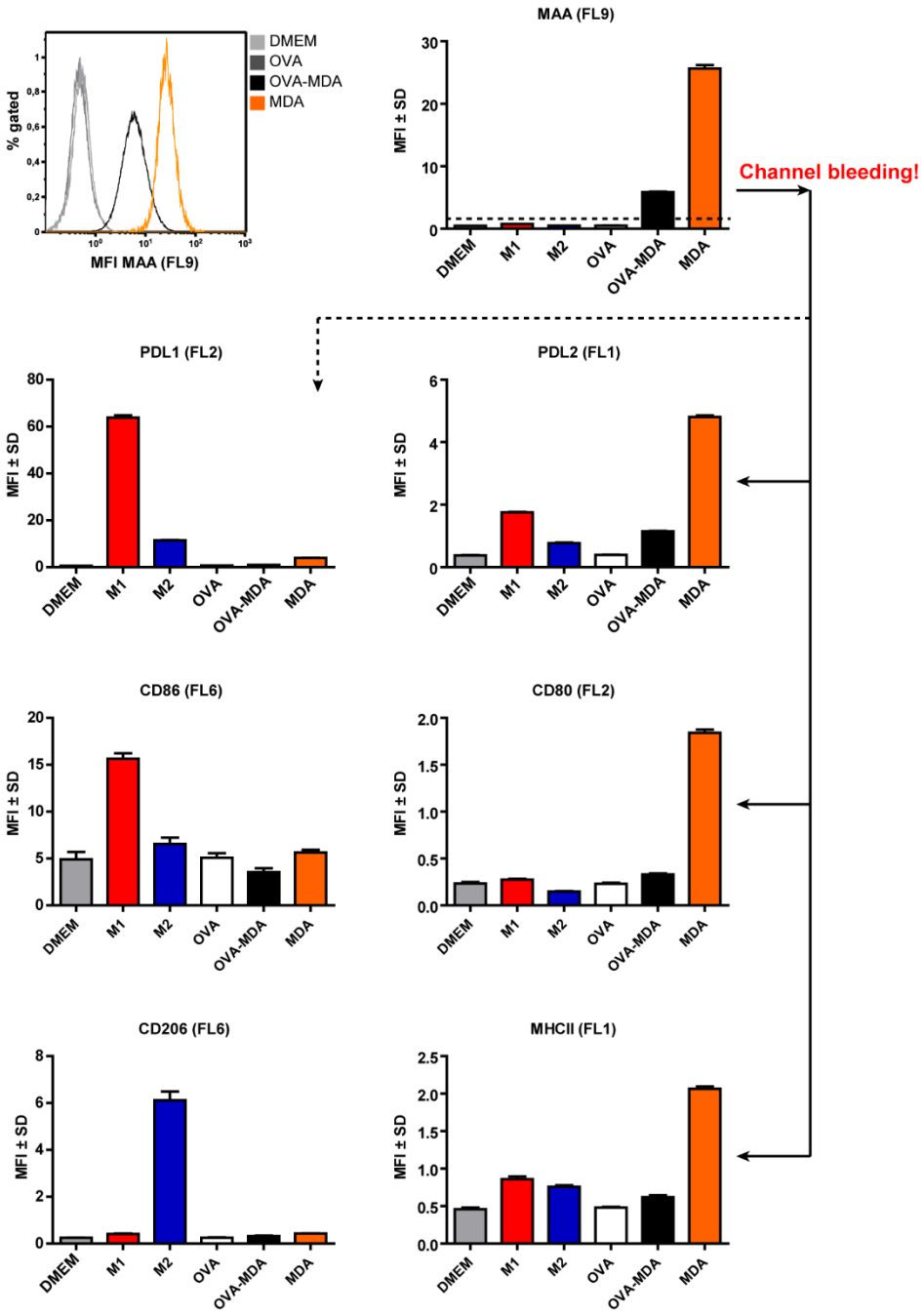
A further notion is that aldehydes can be hydrated, especially at lower pH ranges. Alternatively, aldehydes can also be metabolized by aldehyde dehydrogenases to the corresponding carboxylic acid. Both these mechanisms affect the final nature of respective adducts and analytical methods. In connection to alcohol-induced liver pathology it has been reported that deficiency of aldehyde dehydrogenase 2 worsens inflammation [222].

Lastly, vast alterations to the charge and topology of the carrier protein may cause it to aggregate and precipitate. Precipitation in turn will make it difficult to accurately determine protein concentrations and changes aspect that relate to nanoparticles as opposed to soluble protein.

With all the above in mind it seems impossible claim any specific or homogenous modification with defined MDA adducts. I therefore mostly refer to MDA-modified proteins as a protocol rather than a specific adduct. Furthermore, the difficulties associated with MDA modification are important to keep in mind during the analysis, interpretation of data and especially during experimental preparations.

[54] Relevant channels in the Gallios Flow Cytometer are explained in Figure 7.

Figure 7: Fluorescence of MDA adducts or soluble MDA oligomers interferes with FACS readouts



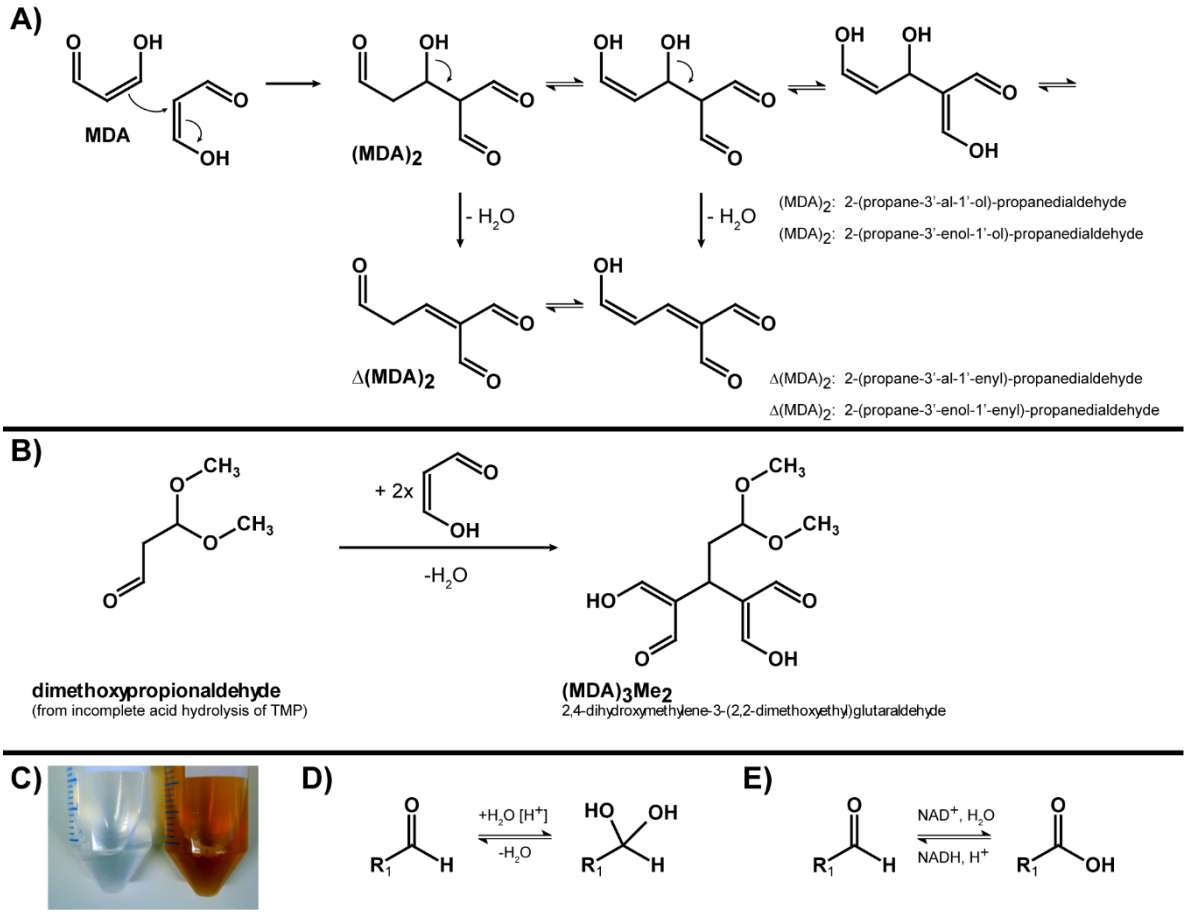
RAW264.7 macrophages were polarized towards M1 (LPS+IFNγ) or M2 (IL-4/IL-10/TGFβ), or treated with the indicated proteins or pure MDA oligomers for 24 h. Cells were prepared and stained for analysis by FACS with antibodies to the indicated targets.

Top left: the FACS histograms for FL9 reveal emission for fluorescent MDA products.
Top right: the median fluorescent intensity (MFI) quantification shows that fluorescence is apparent is associated to MDA modification, but significantly more evident in MDA oligomers used as positive control.
Lower panel: The arrows indicate channel bleeding into mostly FL1, but also FL2. Note that the bleeding effect to FL2 has more impact on targets with low signal intensity (CD80) as opposed to strong signal intensity (PDL1). There is no signal bleeding into FL6. Signal bleeding may inspire false conclusions, e.g. that CD80 would be induced by OVA-MDA, which is not confirmed by RT-PCR (data not shown).

Relevant channels in the Gallios Flow Cytometer:

Channel	Exitation (nm)	Emmision (nm)	Dye example
FL1	488	525/40	FITC
FL2	488	575/30	PE
FL6	638	660/20	Allophycocyanin
FL9	405	450/50	DAPI, Pacific Blue™
FL10	405	550/40	Pacific Orange™

Figure 8: MDA oligomerization



A) Example for two units of MDA oligomerizing. The reaction itself can propagate to include many more units of MDA. Tautomerism accounts for various intermediates of the $(MDA)_2$ molecule, which can further undergo dehydration to create a more extensive delocalized π-system, which in turn accounts for absorbance and color. Different oligomers can continue to react to more advanced products.

B) Another example of MDA oligomerization involving incompletely hydrolyzed TMP.

C) Example of a freshly prepared MDA solution and a reacted one. Note that the fresh solution (left) is transparent and clear, whereas the reacted solution (right) has given rise to red color over time. This color development is independent of the addition of amines.

D) The hydration of aldehydes is acid catalyzed, reversible, and changes the nature of the functional group.

E) Aldehydes can be oxidized by aldehyde dehydrogenases to carboxylic acids.

1.5.3 MDA IN VIVO

1.5.3.1 MDA metabolism and molecular targets

Soluble MDA is metabolized to CO_2 inside the body with an approximate half-life of 4 h [223], although a major fraction is expected to react with available amines directly. Adduction may prolong the half-life of MDA [224]. The major carriers of MDA in vivo are apoptotic/necrotic cells, microvesicles and oxidized lipoprotein particles [175]. A major metabolite of MDA-lysines, (Nα-acetly)-(Nε-propenal)-Lysine, is secreted via urine [205].

The reactive nature of MDA makes it likely to react with the next best amine, thus implying membrane proteins and enzymes as the major targets. Mass spectrometry approaches have been performed in order to identify the targets of MDA modification [175, 225, 226]. Enolase has been identified as one of the many targets, which is interesting as enolase is also a autoantigen in rheumatoid arthritis [166]. While using alternative tissues may identify more targets, a major issue with most Mass Spectrometry approaches (e.g. 2D gel electrophoresis) is an inherent negative selection bias for membrane proteins. In relation to MDA being associated to lipid peroxidation and membranes this implies that many potential targets are possibly being missed.

1.5.3.2 Association of MDA adducts to disease pathology

Lipid peroxidation products are established markers of oxidative stress in the pathology of various diseases. Accordingly, lipid peroxidation has been implicated their respective pathogeneses although direct causality may be difficult to establish. The presence of lipid peroxidation markers is thus reminiscent of a 'chicken-egg' discussion, because the fact that lipid peroxidation is active does not automatically infer that it is the main culprit for pathogenesis. Conversely, neither does it exclude that MDA is relevant to the pathogenesis.

There is convincing evidence for MDA being important in age-related macular degeneration [104] and atypical uremic syndrome [227]. Both diseases have established associations to mutations in complement factor H, a soluble complement component that enhances complement-mediated opsonization but counteracts final complex formation and pro-inflammatory effects (reviewed in [228]). Importantly, factor H binds MDA adducts [104], thus establishing a link to homeostatic clearance of stressed or apoptotic cells. Conversely, MDA adducts are also reported to be recognized by the driving complement component C3 [105] in turn implying a balance between clearance and pro-inflammatory activation[55].

In this context the disease involvement of MDA is established via the association to factor H deficiencies. These have been associated to other diseases such as lupus nephritis [229] or

[55] Notably, tests for direct binding of C3 to MDA were negative using purified components instead of sera (Weismann *et al.*, 2011, supplementary data). Factor H is a known interaction partner of C3, therefore the ELISA for C3 binding to MDA in (Veneskoski *et al.*, 2011) possibly is indirect via factor H. If so, this would further argue in favor of anti-inflammatory recognition and clearance of MDA adducts.

smoking-induced lung cancer [229], possibly implying a contribution of MDA in these diseases.

Based on pathology [230] MDA is associated to a range of other diseases, including liver injury [231], lung injury [232] and epilepsy [233]. In particular, there is an emerging role of MDA adducts in the pathogenesis of atherosclerosis (reviewed in [175, 234, 235]), especially its inflammatory aspects [236]. Briefly, MDA is linked to inflammation in atherosclerosis by the involvement of lipoprotein particles and their MDA-mediated overwhelming uptake into lipid-laden 'foam cell' macrophages. Furthermore, ER-stress [237] and ensuing apoptosis with necrosis of these cells creates a perpetuating inflammatory environment. Lastly, there are several lines of evidence suggesting adaptive immune responses in the form of antibodies directed towards MDA adducts. Together these features support a role for MDA adducts in atherosclerosis [175, 235].

1.5.4 PATHOLOGICAL AND PRO-INFLAMMATORY EFFECTS OF MDA

I will start off this chapter by clarifying my personal opinion that many of the immediate pro-inflammatory effects of MDA are probably attributable to bystander effects associated to cell stress and death and the release of danger-associated-molecular patterns (DAMPs), rather than being specifically and directly induced by MDA adducts. This is largely based on my personal endeavors throughout my PhD in failing to establish clear pro-inflammatory effects of MDA-modified protein. However, the typical dose of 50 µg/mL I applied in my research projects is far below what most publication use in conjunction with effects related to MDA-adducts (typically >200 µg/mL), but the effects I observed on uptake and proliferation were already clear (cf. discussion of Study I).

1.5.4.1 Modification of nucleic acids and mutagenesis

MDA exerts toxic effects to cells starting at doses around 100 nM [205]. This toxicity can be attributed both to protein and lipid modifications, but also to DNA modification [238, 239]. All nucleic acids can be modified by simple MDA-adducted derivatives, but most notably guanine that can be modified by MDA and condensate further to a cyclic-adducted variant called M_1G[56]. [239]. M_1G is an established marker of DNA damage and has mutagenic and carcinogenic effects and furthermore MDA adducts in DNA arrests transcription [240]. Thus

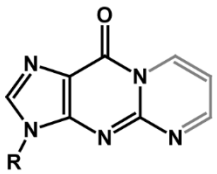


Figure 9: M_1G

Structure of M_1G. The original guanine residue is shown in black. The ambiguous R corresponds to a hydrogen in MDA-guanine, or the sugar backbone in MDA-guanosine residues. The cyclic MDA adduct is shaded grey.

[56] MDA-Guanine (pyrimido[1,2-a]purin-10(3H)-one)

while MDA is toxic to cells and induces DNA damage, this attribute is largely lost if MDA is bound to protein.

1.5.4.2 Release of pro-inflammatory cytokines

A number of studies have reported the release of cytokines in response to exposure to MDA-modified protein.

The human THP-1 cell line induced CCL2, IL-6, CCL11, CXCL13 after stimulation with 50 μM MDA or MDA-lysine [241] and produces IL-8, IL-1β, TNF-α, and IL-12β after stimulation with 50 μg/mL of MDA-BSA cells [104]. IL-25, IL-6 and IL-8 were induced in the Jurkat T cell leukemia line and human peripheral blood lymphocytes treated with MDA [242].

In mice, CXCL1 (KC) gene expression was induced in retinal pigment epithelium (RPE) assayed *ex vivo* 6 h after injection of MDA-BSA into the eye. Mouse bone marrow-derived macrophages in the same study required >100 μg/mL of MDA-BSA to induce ~100 pg/mL of CXCL1 *in vitro* [104].

Rat liver endothelium or Kupffer cells were reported to secrete increased TNF after co-incubation with MDA-BSA at 25 μg/mL and LPS, compared to LPS alone [243]. Similarly rat hepatic stellate cells were reported to dose-dependently secrete CCL2 and CXCL2 in response to MDA-BSA (up to 100 μg/mL) [244].

Personally, I have performed ELISAs for TNF secretion by either mouse bone marrow-derived macrophages (WT and CD36 knockouts) or RAW264.7 cells incubated with 50 mg/mL of MDA-proteins, but observed no induction other than in positive controls. I think it is unclear whether these cytokines are in fact released in response to MDA-modified protein, or rather as a consequence of cellular stress and/or death.

1.5.4.3 Other pathological effects of MDA

Besides pro-inflammatory cytokine release there are a few pathological effects associated with MDA or MDA-modified proteins. For instance, MDA modification has been reported to interfere with the function of hemoglobin [245]. Other studies report MDA-modified proteins to stimulate pro-inflammatory and pro-fibrotic processes [246], vascular injury [247] or damage to lung tissue [232]. Apart from the above notions, there have been implications for MDA adducted proteins challenging lysosomal integrity [248], inducing ER stress and interfering with processing events [249]. The latter issues have been addressed in the context of Study I. Lastly, it is possible that APCs chemically reduce presented aldehyde antigens

[250]. However, it is not established that the adducts themselves actually represent T cell epitopes and their size extension would argue against it.[57]

1.5.4.4 Signaling induced by MDA

Lastly, MDA or its adducts have been implied to induce cellular signaling, although how this occurs is mechanistically poorly elucidated. Activated pathways include protein kinase C γ (PKCγ) [251], mitogen-activated protein kinase 1/ extracellular signal–regulated kinase (MAPK/ERK 1/2) and nuclear factor kappa B (NFκB) [222]. I discuss below that possibly co-recognition by scavenger receptors and TLRs induces signaling. Furthermore, oxidized phospholipids possibly modulate the cellular signaling [252]. The signaling mechanisms of the related advanced glycation end products are reviewed elsewhere [253].

1.5.5 INNATE RECOGNITION OF MDA ADDUCTS

MDA modification profoundly changes the biochemical properties of the carrier protein [202, 254]. In particular the adduction of lysine amines eliminates positive charges and drastically changes the residue's topology. These changes convey properties that can potentially be recognized by innate pattern recognition receptors (reviewed in [255]). Specific recognition of MDA adduct is thus possibly a basis for inducing innate immune responses.

1.5.5.1 Recognition by scavenger receptors

Recognition of adducts associated with oxidative stress is widely accepted to entail recognition by components of innate immunity [183, 255]. This inspires the conclusion that such adducts constitute a novel type of DAMPs [256].

Scavenger receptors are groups of heterogeneous receptors that recognize a wide range of ligands or patterns with implications for clearance and innate immunity (reviewed in [161]). Various MDA-modified proteins have been demonstrated to be selectively phagocytosed by Scavenger Receptor A (SRA) [221, 248, 257-260], CD36 [258, 261] and possibly LOX1 and FcγRIII (CD16) [261]. Importantly, SRA is undoubtedly the main receptor for MDA-adducted protein and I have extensively addressed this in Study I. CD36 is mostly reported as a receptor for MDA adducts in the context of LDL, while CD36 is the prototype scavenger receptor of oxidized LDL (oxLDL) [161]. MDA in turn has been suggested as one dominant component of oxidized LDL [262]. The binding of MDA-LDL by LOX1 and FcγRIII has also been

[57] This is a major issue with MDA compared to small PTMs such as citrullination or acetylation. While the latter can be easily accommodated during antigen presentation, MDA adducts are generally much too bulky and are expected to sterically obstruct MHC and TCR interaction should they be present in the MHC groove. While citrullination alters the charge of former arginine residues, the volume is essentially the same. Conversely, MDA adduct render the modified lysine residue roughly twice as large.

demonstrated, although the relative binding is >15-20 times lower than for SRA (cf. [261]). The FcγIII receptor is important for monocytes [263] and has been implied to be critical for the induction of EAE, although the relative importance differs between independent publications [264, 265].

The ligation of scavenger receptors has been implied to possibly contribute to the maturation of DCs [266]. The complication is that the known scavenger receptors feature very short cytoplasmic tails devoid of classical signaling motifs. Conversely, it is starting to emerge that scavenger receptors utilize or modulate the signaling of other pattern recognition receptors, e.g. TLR4 upon co-ligation [261]. This is very interesting as it opens up new possibilities and implies that the nature of the (modified) ligands influences the cellular responses.

1.5.5.2 Recognition by complement

As discussed above, MDA adducts have been reported to be recognized by complement factors H [104, 227] and C3 [105], although the latter was demonstrated using serum and potentially mediated via factor H. Nonetheless, these studies highlight the innate recognition of MDA adducts and establishes a link to clearance mechanisms, with potential tilting toward inflammation in case the clearance mechanisms are defective or depleted.

1.5.5.3 Recognition by natural antibodies

In addition to the innate recognition mechanisms above, natural antibodies have been demonstrated to recognize MDA-derived epitopes [267-269].

1.5.5.4 Conclusions on innate recognition.

Together these observations imply evolutionary advantages with innate recognition of lipid peroxidation products. This suggests that the mechanisms described above mostly exist to limit potential damage associated with lipid peroxidation and reactive products thereof. Furthermore, this conchords with the association of MDA adducts to cellular stress, death and debris. The common denominator of innate recognition appears to be *clearance*, rather than *inflammation*. Notably, this does not exclude that inflammation could occur in response to non-apoptotic cell death and release of DAMPs if the situation cannot be contained. Lipid peroxidation *in vivo* is very common and inevitable, much as the occurrence of many other PTMs. I therefore think that default pro-inflammatory responses to such events would be detrimental.

1.5.6 MDA AND ADAPTIVE IMMUNITY

In continuation to innate recognition I will discuss adaptive responses to MDA adducts. As discussed earlier there are mechanistic connections that imply a role for MDA in the pathology of many inflammatory diseases involving adaptive immunity.

It appears that MDA modification has gained the status of a hapten [270] that converts the carrier antigen to a 'superantigen' of sorts. This is based on a series of studies reporting that MDA-adduction breaks immunological tolerance [271, 272]. Typically, elevated T cell responses [221, 273] and levels of autoantibodies [274, 275][58] are reported as a consequence of MDA-adduction to the carrier protein. The climax is the claim that MDA-modified proteins are immunogenic *per se* in the absence of adjuvant [276].

However, there is a flip side to the coin, namely that adaptive immune resonses towards MDA potentially constitute 'protective autoimmunity' [277] in a sense that it augments clearance mechanisms discussed further above. There are several studies implying that MDA-directed adaptive immunity is protective in atherosclerosis [278-282], although this is challenged by anti MDA-LDL antibodies being suggested as a potential negative predictive marker in cardiovascular disease [283]. Together these studies call for approaches to establish standardized methods for the reliable detection of antibodies recognizing MDA adducts [284], and the contextual investigation of adaptive immunity [175] in order to clarify the role of MDA adducts and antibodies towards them.

We currently understand that adaptive immune responses to MDA adducts are dependent on $CD4^+$ cells [285], MHC class II and functional TCRs [286]. Advanced glycation endproducts (AGEs) and advanced lipoxidation endproducts (ALEs) notably share many common features [183] and antibody cross-reactivities have been identified [287]. Furthermore, there is cross-reactivity between MDA antigens and bacterial antigens [288].

Future studies will be required to elucidate the specificities and involvement of adaptive immunity towards lipid peroxidation products.

1.5.7 REFLECTIONS ON MDA

In conclusion, I have tried to review the current knowledge regarding MDA and its adducts from my personal (flavored) perspective. In light of the abundance in literature about the many properties of MDA or its adducts, major criticism towards some of the conclusions may appear unwarranted. I am nonetheless concerned that certain pro-inflammatory effects attributed to MDA adducts are not necessarily well established, as there is implicit evidence that these may be artifcats related to soluble MDA. However, neither do I present conclusive evidence to the contrary, nor can these processes be dissociated *in vivo*, but there are arguments to be made about the doses. Adaptive inflammatory immune responses towards carrier proteins of lipid peroxidation would bear a serious risk for the development of autoimmunity, in analogy to firefighters using oil instead of water. This assumption has been the reasoning behind the conception of my original study plan. Conversely, articles related to uptake by scavenger

[58] Duryee *et al.* (2008) suggested that MDA-modification could even be exploited in tumor immunotherapy.

receptors and clearance mechanisms agree with my own results and make significantly more sense from a homeostatic biological perspective. My personal experiences compel me to taking this standpoint but likewise make me dismiss previous conclusions. Though this is based on own results one may rightfully question my objectivity on this matter. Hopefully future studies may eventually address some of the concerns and shed light on the discussion about what the effects of MDA adducts truly are.

2 ETHICAL CONSIDERATIONS

Ethical considerations and statement are becoming more common in scientific publications. However, in many cases the focus is essentially limited to animal experimentation and the discussion continues until people arrive at the conclusion that animal experimentation is irreplaceable and should be performed according to humane guidelines while using adequate alternatives wherever possible. The latter is my summary on that matter. I have done everything by the book and have a clear conscience because I considered every possible replacement, reduction and refinement to abide by ethical principles. Animal experimentation only applies to a minority of scientists and is a special scenario. I would rather discuss more universal ethical considerations related to research and publishing ethics.

Every scientist has a personal responsibility to design, perform, document, analyze, interpret and communicate their research in a fashion that will ultimately support valid conclusions. Sustainable science is thus indirectly a product of practiced ethical philosophy. In reality it may be harder to avoid the pitfalls of bias and wishful thinking, but that also requires the awareness to recognize them. Conviction carries the hidden danger of accepting results that support the prevailing hypothesis and dismissing results to the contrary, therefore blinding and critical evaluations ought to be applied more often. Self-criticism defines the scientist.

In modern research the demands to securing funding and publishing have increased significantly, leaving little room for failure. I perceive the ‘publish-or-perish’ pressure to promote a culture in which newsworthiness is valued over quality, and positive results valued over negative ones. However, negative results do not necessarily equal inconclusive results and personally I have learnt a great deal from experiences and experiments that did not work out as expected. I approached my study plan with the conviction that I would gather evidence to prove the concept, but gradually changed my attitude as I grew more confident in my own conclusions that were in disagreement. Science in general always appealed to me because it is based on reasoning and logical principles in the pursuit of knowledge and truth. This ideal was shattered by the reality that ultimately there are only results and conclusions, some more solid than others. In this continuous discourse of evolving hypotheses applied scientific ethics really matter. Dynamic hypotheses can only be shaped based on valid data and if there is no conflicting political interest favoring a dogmatic interpretation. Only prevailing against long-term independent and objective scrutiny lends a hypothesis increasing credibility. A pivotal basis is thus performing honest science with dedication to detail in every aspect and to the best of capabilities and circumstances. There are many approaches to research, but striving for perfection is the only way to do science.

3 DISCUSSION OF STUDIES

This section is dedicated to the more general discussion of the studies. In order to avoid redundancy to the existing introductions and discussions of the individual studies, I intend to give a wider background and perspective in addition to the independent studies. I chose not to review methods in the thesis because it felt redundant to the individual studies and would not have reached a sufficient level of detail to allow for an enlightening discussion. Instead I attempted to mention the motivation and caveats of certain approaches along the way. Below is a condensed overview of the respective studies.

Study I	**"Scavenger Receptor A mediates the clearance and immunological screening of MDA-modified antigen by M2 type macrophages"** **Aim** Assessment of the immunogenicity and uptake of MDA-modified MOG **Methods** Biochemical methods, FACS, ELISA, *in vitro* cultures and EAE **Results** Using fluorescently-labeled MOG and MOG-MDA it is demonstrated that enhanced uptake of MOG-MDA is mediated by SRA and associated with M2 macrophages. *In vitro* proliferation of 2D2 cells is maximized by enhanced uptake of MOG-MDA but EAE induction is equivalent. **Conclusion** MDA-modified antigen is mainly cleared by M2 macrophages via SRA but is also presented to T cells
Study II	**"PyTMs: a useful PyMOL plugin for modeling common post-translational modifications"** **Aim** The creation of a simple tool for molecular modeling of PTMs **Methods** Implementation of a plugin for PyMOL and concept testing **Results/ Conclusion** The PyTMs plugin can be successfully used to address research questions relating to PTMs with certain caveats.

Study III	**"Nitration of MOG diminishes its encephalitogenicity depending on MHC haplotype"** **Aim** Characterization of the immunogenicity of nitrated MOG **Methods** Biochemical methods, molecular modeling, FACS, *in vitro* cultures and EAE **Results** Y_{40} is a critical anchor residue occupying the p1 pocket during presentation by H2-IA^b. The APL nitrated at Y_{40} is predicted not to be presentable due to its structural incompatibility with the p1 pocket. Nitrated MOG abrogates *in vitro* proliferation of 2D2 splenocytes and is not encephalitogenic in the EAE model for C57BL/6 mice, but is fully encephalitogenic in DBA1 mice. **Conclusion** Nitration of the critical anchor residue Y_{40} denies presentation of the MOG_{35-55} epitope by H2-IA^b in C57BL/6 mice, but the MOG_{79-98} epitope in DBA1 mice is unaltered and retains fully encephalitogenic. Nitration is a thus an MHC-dependent mechanism through which antigen-specific T cell responses can be negatively affected.
Study IV	**"Rat bone marrow-derived dendritic cells generated with GM-CSF/IL-4 or FLT3L exhibit distinct phenotypical and functional characteristics"** **Aim/Methods:** The functional and phenotypic aspects of rat bone marrow-derived dendritic cells (BMDCs) generated with either GM-CSF/IL-4 (G4) or FLT3L (FL) were compared using FACS, RT-PCR, Cytokine Bio-Plex and T cell proliferation assays. **Results/ Conclusion** G4-BMDCs excel at phagocytosis and express CD11b and CD11c, whereas FL-BMDCs express CD103 and CD4. FL-BMDCs have a stronger upregulation of class II and co-stimulatory molecules upon activation and induce antigen specific T cell proliferation and skewing towards Th1 and Th17 subsets, unlike G4 that promote IL-10-producing Tregs and induce poor T cell proliferation. The BMDC cytokine profiles differ. The respective BMDCs are functionally and phenotypically distinct.

3.1 STUDY I: "SCAVENGER RECEPTOR A MEDIATES THE CLEARANCE AND IMMUNOLOGICAL SCREENING OF MDA-MODIFIED ANTIGEN BY M2 TYPE MACROPHAGES"

3.1.1 HISTORIC CONTEXT OF STUDY I

The design of this project was originally an entirely different one. According to my study plan I was supposed to investigate the use of MDA-modified MOG as a tolerance-inducing vaccine [289]. There are two underlying studies performed by a former PhD student in the lab several years before me that I will need to introduce to give a background [272, 289]. I will do so by summarizing the essential data according to the original figures, but I do recommend reading them separately. The focus in on the study related to MDA [272].

"Vaccination with myelin oligodendrocyte glycoprotein adsorbed to alum effectively protects DBA/1 mice from experimental autoimmune encephalomyelitis" [289].

Figure 1	An ALUM-based vaccine preparation of MOG protected DBA1 mice from EAE if injected in advance
Table 1	The above only worked i.p.; MOG-IFA conveyed the same effect.
Figure 2	The injection MOG-CFA s.c. evokes T cell responses in inguinal lymph nodes, but MOG-ALUM i.p. does not; both induced equal responses in mesenteric lymph nodes. Prior vaccination with MOG-ALUM followed by a challenge with MOG-CFA increased mesenteric responses, but reduced inguinal responses. Splenic responses were equal.
Figure 3	MOG-ALUM vaccination prevented the migration of CD4+ or CD4+CD25+ T cells into the CNS
Figure 4	MOG-ALUM vaccination is characterized by a reduction of MOG-reactive IgG2a and an increase in IgG1
Figure 5	CD4 knockout DBA1 mice are not protected by MOG-ALUM vaccination[59]
Figure 6	An analysis by RT-PCR of *ex vivo* re-stimulated T cells detected reduced IFNγ and IL-10 from vaccinated mice

The mechanism by which this supposedly induces tolerance is unclear. In modern approaches I would probably look at the induction of Treg cells or anergy. What puzzles me is that the vaccine only works i.p. but not s.c. and targets different lymph nodes than the s.c. CFA-based EAE induction. I did perform a larger experiment using s.c. vaccination[60], but that did not confer protection in C57BL/6 mice.

[59] I'm surprised these mice develop EAE
[60] Our current ethical permits did not allow for i.p. immunizations

"Malondialdehyde modification of myelin oligodendrocyte glycoprotein leads to increased immunogenicity and encephalitogenicity" [272]

Figure	Reported data	My concerns
1	MOG-MDA has a lowered isoelectric point. Mass Spec detects simple MDA adducts at K_{30}, K_{80} and K_{114}, but not K_{55} or K_{73}. A corresponding 3D model is presented.	• The presented model used simple N-propenal lysine MDA adducts, although Mass Spec will additionally identify advanced MDA adducts if they are included in the search. • K_{55} is the most accessible lysine residue in MOG and is normally modified (possibly cross-linked), K_{73} is inaccessible though. • The display with electrostatic surface potential is misleading because the electrostatic map does not apply to the modified residues, making them appear white. • A further errata is that the article reports a more *positive* charge by MDA modification, although this should correctly be *negative* instead, especially if the pH is above the pI (which it is). • Rat MOG precipitates and aggregates heavily by MDA modification.
2	MOG-MDA, but not MOG, evokes fatal EAE in DBA1 mice	• I failed to reproduce this observation using mouse MOG both in C57BL/6 and DBA1 mice in blinded experiments.
3+5	Enhanced T cell proliferation towards MOG-MDA, inhibitable by poly I:C	• I could fully reproduce this phenotype using 2D2 cells *in vitro*, but not with *ex vivo* lymph nodes from immunized mice (probably due to the low frequency of specific T cells). Study I confirms that the enhanced proliferation is due to uptake by SRA. Minor: although used as a competitor here, poly I:C is a TLR3 ligand.
4	Higher uptake of MOG-MDA by macrophages, detected by intracellular His-tag staining and flow cytometry	• The supplementary data of Study I demonstrates that the intracellular His-tag staining produces artifacts. This method fails to reliably detect MOG inside cells and severely underestimates the degree of uptake. The minor differences observed in the reported figure possibly relates to channel bleeding (cf. Figure 7, pg. 40).
6	Induced gene expression of IL12R, IL-12 p35 and IL-23 p19 in macrophages in response to MOG-MDA	• I have tried to detect increased cytokines by ELISAs and RT-PCR approaches without success.

3.1.2 DISCUSSION OF STUDY I

3.1.2.1 Challenges concerning mouse MOG production

My initial challenge was to produce mouse MOG and the MDA modification for EAE induction. The older preceding studies used rat MOG and DBA1 mice, but we were to transition to mouse MOG and C57BL/6 because this strain is more commonly used and offers a significantly wider variety of transgenic models and tools. Secondly, there is no justified motivation to use rat MOG in mice as this may entail artificial mechanisms due to sequence differences (discussed in Study I). In essence, the goal was to establish a working model.

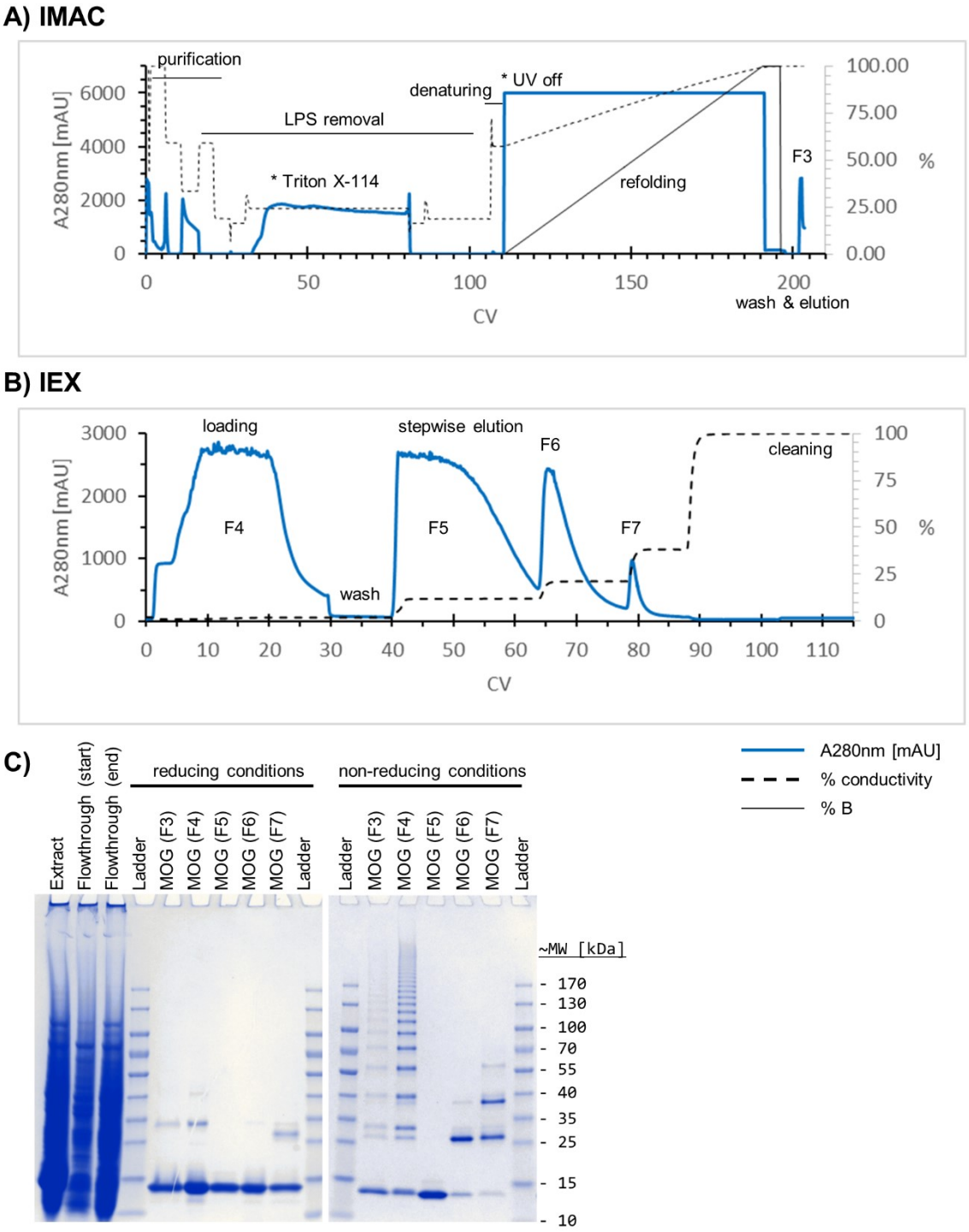
My initial problem was that the production of recombinant MOG did not yield pure enough protein and contained aggregates. Since one aim was to compare the immunogenicity I wanted to avoid any artifacts related to bacterial contaminants or protein aggregation. A second motivation was to optimize both yield and purity because at the time one of the study goals was to possibly resolve a crystal structure of MDA-modified MOG. Over time I invested extensive efforts to polish the MOG production, which was eventually upgraded from a very labor intensive and time-consuming manual process to a standardized automated production. By joining His-tag affinity chromatography with an ion exchange chromatography this method is able to specifically isolate monomeric folded protein and get rid of misfolded and aggregated protein. The process as a whole has been at the heart of my PhD studies and is depicted in Figure 10, but is too extensive to describe here in full detail.

The automated production of monomeric mouse MOG is a multistep process involving two main methods: Immobilized Metal Affinity Chromatography (IMAC) and Ion exchange chromatography (IEX).

A) The recombinant His-tag binds immobilized Ni^{2+} ions on the IMAC column. Various impurities are removed in a series of steps involving different elution conditions. Subsequently residual endotoxins such as LPS are removed by two approaches (precipitation and extended elution with Triton X-114). The column is equilibrated, the protein denatured again and allowed to refold in place. After final washing steps, the fraction F3 is eluted (purified MOG).
B) The F3 fraction is directly loaded onto and IEX column for further removal of contaminants and polishing. The F4 fraction does not bind the column, F5-F7 are eluted after washing with increasing amounts of salt.
C) Analysis by SDS-PAGE and Coomassie staining with either reducing or non-reducing conditions. Under reducing conditions MOG yields 1-2 bands implying a high efficiency of purification from the extract. However, reducing conditions reveal that F3 still contains misfolded multimers of MOG that are sensitive to reduction. These multimers are separated by IEX into F4 (aggregated column non-binding), F5 (monomeric MOG, one band!), F6 (mostly dimers) and F7 (mostly trimers). F5 is the final product used for experiments after dialysis to PBS.

CV=column volumes. Note the insert legend for the graphs. Asterisks indicate the reason for unexpected absorbance. Fractions (F) are indicated.

Figure 10: Production of purified monomeric mouse MOG



3.1.2.2 Challenges concerning MOG-MDA and the EAE phenotype

Several analyses reproduced the biochemical features of MOG-MDA, but also led to a more diversified approach in respect to the advanced MAA adducts. I quickly recognized that the homogenous modification with MDA is a myth due to the lack of control over the chemical reactions, but also that the instability and heterogeneity would not be suitable for clinical applications, i.e. vaccines. If anything, one would require defined and stable modifications that mimic the effects of MDA.

A very consistent result was that MOG-MDA did enhance the proliferation of 2D2 splenocytes. The fact that we observed increased proliferation *in vitro* always motivated the expectation that the EAE phenotype should be more severe. However, I failed to produce evidence that MOG-MDA in fact evokes more severe EAE. The challenge in this respect is that the EAE model is already very harsh and artificial. I theorized that if MOG-MDA was indeed more immunogenic, there should be conditions at which it evokes EAE, but not native MOG itself, but it is almost impossible to identify such a condition in the EAE model.[61]

I have tried immunizing both C57BL/6 and DBA1 mice with variable doses of MOG and different adjuvants (IFA, CFA with variable doses) without noting differences as clear as they were previously reported [272] (cf. Study I and Figure 11C). After the initial experiments I went over to blinding myself for all in vivo experiments to minimize my personal bias. Issues related to the use of MDA-modified rat MOG are discussed in Study I. Briefly, the MOG_{79-98} epitope in DBA1 mice [115] contains an amino acid substitution[62]. It was demonstrated for the human MOG_{35-55} epitope[63] in C57BL/6 mice that the EAE is instead driven by B cell-dependent mechanisms and requires a secondary response to endogenous MOG [32, 122]. This may imply that MDA adducts possibly enhance this process, possibly by overriding the necessity for MOG-specific B cells with additional MDA adduct-specific B cells, although this is pure speculation.

Apart from the regular amino acid substitutions between species the rat MOG construct features additional amino acid extensions on both the N- and C-terminus and uses a different linker to the His-tag [290], which confers alternative biochemical properties compared to the mouse construct. In relation to this a second aspect is that MDA modification causes precipitation of both rat and human MOG (cf. Figure 11B, pg. 61). The original article states: “Protein was separated from unreacted free aldehyde by two rounds of dialysis against PBS. The precipitate was removed through centrifugation […]” [272]. For me this sentence implies two things: firstly, that the two rounds of dialysis were insufficient to reliably remove soluble MDA, and secondly, that the protein was heavily aggregated. Micro-aggregates relating to the latter cannot

---

[61] Imagine you wanted to show that there was a difference in temperature between tea and iced-tea, but would have to throw the cups into a burning volcano to measure the temperature. Replace the teacups with MOG/ MOG-MDA and the volcano with CFA and you will be essentially looking at the problem with the EAE model.
[62] GKV**T**LRIQNVRFSDEGGY, rat: 82=**A**, mouse 82=**T**.
[63] MEVGWYR**S**PFSRVVHLYRNGK; human 42=**P**.

be reliably removed by centrifugation, either. These may cause altered immune recognition and make it difficult to reliably determine the protein concentration, probably underestimating it. The fact that a considerable fraction of protein is lost to the precipitate lowers the concentration and, accordingly, the dilution factor for contaminants[64] (discussed further below). Lastly, it is unclear how representative the soluble fraction of rat MOG-MDA is of the entire preparation.

3.1.2.3 A reflection on protein doses used in EAE

A mouse brain weighs approximately 0.5 g wet weight, the brain protein content is <10%, MOG is ~0.05% of myelin protein. That yields: $0.5g \times 10\% \times 0.05\% = 25\mu g$

In other words, if I immunize a mouse with 50 µg of MOG, there is roughly twice as much MOG in its tail base than in its entire brain. Exposing 2D2 cells to 50 µg/mL of MOG corresponds to isolating all MOG from one mouse brain and diluting it in just 1 mL. The point is that these experiments are already highly artificial. Dialing up the dose in hope of effects would simply add to the existing artificialness.

3.1.2.4 Chicken-egg. Soluble MDA or MDA adducts – can the effects be dissociated?

I intended to perform a proliferation assay with 2D2 cells using freshly modified MOG-MDA, so I modified MOG with MDA for 3 h and subjected it to several rounds of dialysis against PBS for ~2 h each while preparing the 2D2 splenocytes in parallel. According to the manufacturer of the dialysis cassettes complete buffer exchange is achieved within 2 h, but this is based on the dialysis of salt using membranes with significantly larger pores. All 2D2 cells exposed to MOG-MDA died in the experiment. For a subsequent repeat I had re-dialyzed the same batch along with a more properly dialyzed independent batch. Titration experiments revealed the typical increased proliferation towards MOG-MDA over MOG, but also a remaining toxicity at higher doses (Figure 11C). My conclusion from this experience was that MDA toxicity exists in preparations of MDA-modified protein, and has to be removed extensively if the aim is to address the effects of the adduct without over-layering effects of soluble contaminants. I subsequently invested some efforts using tracers to optimize the dialysis step, which revealed that establishing equilibrium takes several hours (~6 h), and that dialysis needs to be performed over several equilibria (>4x) to reliably remove contaminants.

The above rendered me skeptical towards reports attributing certain effects to MDA adducts as opposed to the possibility that soluble contaminants may also account for them. It struck me that the majority of reports on the pro-inflammatory aspects of MDA were authored by the same core group of authors, implying a lack of independent reproduction. Startlingly, some of these studies report the toxicity of MDA-modified proteins in vitro at above 100 µg/mL [221][65], but they immunized animals with a dose corresponding to 125 µg/mL. Subsequently they observe elevated immune responses towards MDA-modified antigen, but conclude that

---

[64] A lower protein concentration will require more input to achieve the same concentration.
[65] Simply truncating data in the corresponding graphs.

this is attributable to the MDA adducts alone [221, 276], while cell death is either ignored or attributed as a specific effect of adducts [248]. Conversely, I failed to detect lysosomal instability [248] (cf. Figure 11E) or synergy with LPS for TNF secretion [243] with MDA-modified protein. Personally, I went from accepting the above-mentioned conclusions as fact to seriously questioning their validity. Applying *Ockham's razor*, the simple explanation is that MDA-induced cell stress and death result in the release of DAMPs, accounting for the immune stimulation.

Accordingly, a new open question arises: is it even possible to dissociate the effects of lipid peroxidation and antigen modification *in vivo*? After all these processes can be separated in biochemical research, but will occur indiscriminately *in vivo*. There is no definitive answer to this question, but *in vivo* there are more factors involved, yet the concentrations of MDA are also expected to be significantly lower.

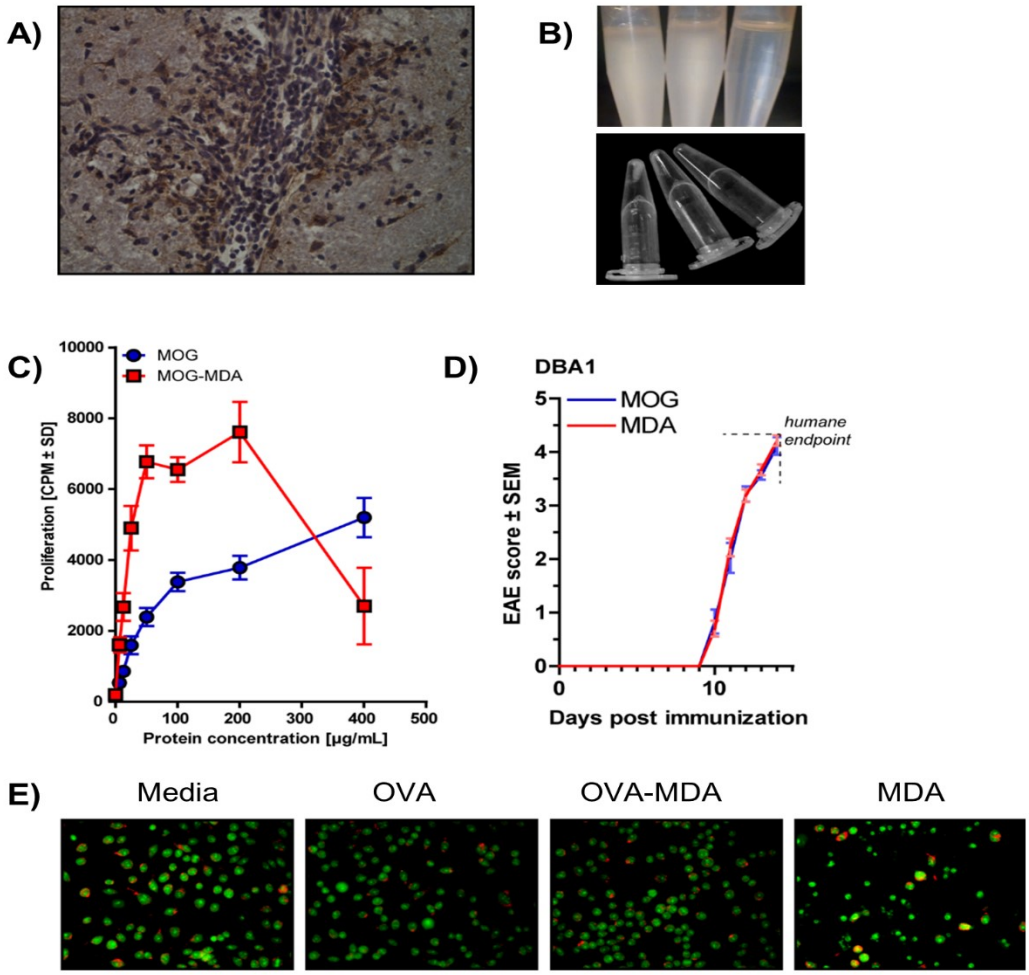
3.1.2.5 Uptake and M2 macrophages – a role for the innate recognition of MDA adducts

One centerpiece of Study I is the uptake by SRA. Although the approach is very elegant and conclusive I should point out that this is not a novel discovery. Critics may rightfully argue that this is just a fancy way of demonstrating what we already know. However, the novel aspects are the association to M2-type macrophages [291] and effects on digestion.

The notion that innate recognition of lipid peroxidation products by SRA primarily associates to macrophages with homeostatic phenotypes agrees with the concept of clearance and tissue repair. It also explains why no differences are to be expected in the priming of EAE, since classical DCs do not express SRA. However, it somewhat disagrees with the *in vitro* proliferation results of 2D2 mice. One simple explanation could be the abundance if protein suffices for presentation by all APCs and induces *in vitro* proliferation regardless. The fact that blocking SRA actually reduces proliferation would argue against this. A potential consensus would be that although the antigen is effectively taken up, it is also is presented for potential recognition by memory T cells, in case the tissue damage is in fact associated to a pathogen. Furthermore, different populations of APCs possibly respond in a variety of ways to identical stimuli [292-294].

The observation that MDA adducts interfere with digestion of carrier antigens seems expectable, but has important implications. Although I did not identify any reason to believe the digestion of MOG by macrophages would be affected, this may differ for other antigens. It could furthermore prolong the half-life of protein debris in the extracellular space. I wonder whether a potential inability to process certain cargo may contribute to the phenotype of ‘foam cell’ macrophages in atherosclerosis, besides the overwhelming uptake of lipids associated with oxLDL. The inability to handle the immense amount of cargo is one factor that possibly leads to metabolic stress and non-inert forms of cell death of these cells, which contributes to the inflammation in atherosclerotic lesions.

Figure 11: Additional data related to Study I



A) Immunohistochemical staining of MDA adducts (brown) in a section of a CNS infiltrate in EAE spinal cord. The tissue is stained with hematoxylin. This staining implies that MDA is associated with CNS inflammation.

B) Precipitation of human and rat MOG by MDA modification. recombinant human, rat an mouse MOG (tubes left to right) were modified using MDA. Unlike mouse MOG, human and rat MOG developed immediate turbidity (upper picture). Centrifugation of aliquots precipitated macro-aggregates in human and rat, but not mouse MOG (lower picture).

C) Thymidine incorporation assay of 2D2 splenocytes using two batches of MOG and MOG-MDA that were insufficiently dialyzed. Proliferation is enhanced by MOG-MDA at doses from 25-200 µg/mL (due to increased uptake as I demonstrate in Study I). However, at excessive doses the hidden toxicity of soluble MDA becomes apparent. High doses of extensively dialyzed OVA-MDA did not induce cell death (data not shown).

D) EAE in DBA1 mice using MOG or MOG-MDA(25µg in CFA). Note that the batches differ from C and were dialyzed.

E) RAW264.7 cells were exposed to the indicated proteins or soluble MDA for 24 h and stained using acridine orange (AO). The fluorescence of AO is pH dependent, and it stains acidic lysosomes red and develops green fluorescence when bound to RNA/DNA. The analysis revealed that lysosomes were intact in controls and protein-treated cells, but displayed evidence of disintegration (cytoplasm acidification, yellow stains) along with evident cell death and debris. My interpretation is that soluble MDA accounts for lysosomal instability and cell death, but not its adducts.

In summary, a unified scenario would be that MDA-adducted protein constitutes a clearance signal for SRA on M2 macrophages that initiate tissue remodeling and repair in response to lipid peroxidation. Simultaneously, the debris is processed and presented in order to allow surveillance against possible pathogens, or otherwise recognition of self-epitopes by Tregs. However, should these macrophages become activated by additional stimuli in the surrounding, i.e. DAMPs or PAMPs, this would also upregulate their ability to respond to infection and propagate inflammation if required. This study also raises questions about the reactions of different monocyte/macrophage populations to lipid peroxidation products *in vivo* [292, 295].

3.1.3 WHAT COULD HAVE BEEN DONE DIFFERENTLY?

In retrospect I would do many things differently concerning this work. Mainly I would not run into many of the obstacles I had to learn to circumvent and deal with, yet that was a great part of the learning experience. I question whether I would embark on this project again at all, or rather do something entirely different.

If it had to be this project I would come in with much more criticism to the inherited study plan. Some of the experimental procedures would be performed differently, but I regard those as evolutionary with small improvements or conscious changes contributing to increasingly solid experimentation.

It would be interesting to replicate certain experiments that I have criticized above. I have most issues with the claim that MDA adducts are immunogenic *per se* [221, 276]. By performing similar experiments but controlling for the amount of soluble MDA in the preparations, I could possibly shed light onto the mechanism and formally establish a functional dissociation of soluble MDA and its adducts. I could have also immunized DBA1 mice with suboptimally dialyzed MDA-modified rat MOG for EAE, but I did have one occasion where I added soluble MDA to an emulsion of MOG that developed severe EAE (data not included). Although some of these experiments are feasible to perform, I am uncomfortable with potentially initiating an upsetting political climate. Pointing fingers implies that three fingers are pointing back at me and although we aspire to produce flawless science, we are human and make honest mistakes. Therefore, I believe that discussing these aspects rather than being aggressive about it is the better option, and researchers should establish their own opinion on the matter.

With regards to Study I some it would be interesting to elucidate the transcriptome and signaling networks of various SRA-expressing cells in response to stimulation by either protein, MDA-modified protein, or MDA alone (cf. [292]). This would give unbiased insight into the gene expression profiles and processes in response to these stimuli. Respecting the notion that SRA ligation possibly modulates various responses of TLRs via co-recognition of the same antigen and complex formation, it would be interesting to address this using linked ligands with both MDA adducts and e.g. LPS.

Lastly, if it had to be the EAE model it would be interesting to evaluate a new mouse model. Since double transgenic mice with both MOG-specific TCRs and BCRs develop spontaneous EAE [32]. The ultimate link to lipid peroxidation having a role in pathogenesis would be if transgenic mice with MOG-specific TCRs and an MDA adduct-specific BCRs would display a similar phenotype. This is a risky venture involving the generation of a transgenic BCR mouse followed by additional breeding without the guarantee of success. However, if these mice, but not respective controls, displayed an EAE phenotype it could potentially establish a clear link between lipid peroxidation and CNS autoimmunity.

3.1.4 Conclusions for Study I

The scientific conclusions are stated above and in the study itself. This section has focused on covering the circumstances surrounding Study I and various challenges connected with it. For me this study is more about my personal development as a scientist. It marks the transition from a naïve master student struggling to obtain results that conform to the dogma of the field to having the confidence to shape it, based on qualitatively solid experimentation. In that respect I stood my ground and prevailed as a scientist.

Concerning the immunological effects of lipid peroxidation and MDA adducts there is still much to be discovered. Lipid peroxidation is a pathological process and requires containment by the immune system to avoid propagating tissue damage and bystander inflammation owing to uncontrolled cell death. In this context the extent of lipid peroxidation *in vivo* and limiting antioxidant factors along with innate immune mechanisms form a complex interplay. However, I question that MDA adducts are in fact the '*magic sprinkle*' that convert self antigens into tolerance-breaking '*superantigens*' of sorts by default. If so this would pose a tremendous risk of autoimmunity, lipid peroxidation being such a common phenomenon. MDA adducts and other PTMs have hence been generally demonized and deserve a more diversified point of view. The individual interplay of PTMs in autoimmunity is significantly more complicated than that, and we have just seen the tip of the iceberg.

3.2 STUDY II: "PYTMS: A USEFUL PYMOL PLUGIN FOR MODELING COMMON POST-TRANSLATIONAL MODIFICATIONS"

3.2.1 CONTEXT OF STUDY II

This study also has its roots in the original study plan with a stated objective to resolve the crystal structure of MDA-modified MOG. This never happened for several reasons: Obtaining the large quantities of purified MOG and growing crystals for X-ray crystallography is technically feasible, but this is not possible for MOG-MDA. The major obstacles are that the modification with MDA is not homogenous, that the adducts are flexible and that they introduce crosslinks. A significant problem is furthermore the significant drop in yield of MDA-modified protein connected with approaches to re-purify specific fractions. Lastly, there is no specific scientific question connected to resolving the crystal structure, other than the topology. Given that there is a disproportionate amount of investment required to even attempt crystallization with significant chances of failing, it was not worth pursuing.

At the same time I was introduced to PyMOL molecular graphics software and started figuring out the vast possibilities it offers. Using the basic function I started to manually build and model MDA adducts onto the crystal structure of MOG. This quickly made it apparent that the MDA adducts offered significantly more variety than previously acknowledged, especially when also considering advanced MDA adducts. The second realization was that it was ineffective to do this manually every time, and that it would be easier to reproduce on a different protein if the process were to be automated. That led to me developing a small script for simple MDA adducts. The script was expanded to include customization of the adducts, it grew more complex and intelligent and before long I had a custom function that could introduce MDA adducts on any protein.

Why stop there? By applying the same principles I could make separate functions for other modifications. Later I bundled those into one plugin called PyTMs[66]. As I encountered challenges along the way this project further evolved to use a common infrastructure, deal with chirality, optimize positioning of larger residues, select only surface-accessible residues, perform co-modifications along with various other smaller improvements.

Since I was applying this a lot in order to play around with 'what-if' scenarios, the ultimate application would be connected to my projects in predicting the extent and possible impact of a given PTM. Since the plugin was applicable to give more insights into structurally-related questions, I figured that other researchers in my situation would benefit from it. The PyMOL community is generally very open about sharing scripts and information publically. From there it was then made more user-friendly, and introduced a user interface and other additions to create an independent plugin.

[66] In analogy to PyMOL and PTMs

3.2.2 DISCUSSION OF STUDY II

Study II speaks for itself, so there is not much more to add. However, the really interesting aspects are hidden in the source code[67], namely how to approach and deal with certain challenges related to constructing PTMs and especially sterical optimization aspects.

For instance this involved problems with calculation times that would increase exponentially in order to optimize the positioning of a residue. With some optimizations of the initial concept several weeks of predicted calculation could be reduces to hours or minutes. I was considering to take up some of the details of different approaches, but I can sum it up by listing that the plugin will among other things scan larger intervals and interpolate only the optima[68], calculate the strain locally rather than for the entire objects[69], test initial preferences based on the residue without modification[70], and add or include adjacent objects to account for or not (e.g. waters or another protein).

I do not want to go into too much detail on the above because, despite being a useful functionality, it does not work the way I would want it to. Currently the optimization is residue-based and can move the modified residue around into the optimal position. What I ideally would want it to be capable of performing is a holistic optimization of the entire protein instead.

The elephant in the room is that PyMOL cannot really handle charge or protein optimization adequately. Current molecular dynamics simulations are performed using other software tools that are being developed independently for various purposes. The brick wall that one is running into when coming from the preclinical research side is the translational gap to structural bioinformatics and the steep learning curve associated with it. Especially the necessity to adapt to an entirely new field and the often extremely user-unfriendly tools pose considerable challenges. One of the intentions with the PyTM plugin was therefore to make it user friendly. Below I will outline some of the future challenges connected with Study II.

3.2.3 REMAINING CHALLENGES

3.2.3.1 Port to Python 3

PyMOL is built on Python 2.8 but still being developed. Though no definitive date has been announced future PyMOL versions will be upgraded to Python 3. Currently it is already possible to import selected function from the future Python version, but the intent with this is to give people time to adapt programs and scripts instead of facing an abrupt transition. The important caveat is that Python has changed part of its syntax for certain functions, which is a

[67] The plugin is open source and can be obtained from the repositories indicated in Study II.
[68] Larger steps and interpolation reduce calculation time significantly by reducing the number of calculations, this can still be defined manually for increased accuracy with a time tradeoff
[69] Local optimization saves a lot of time, the more the larger the object, but was tricky to implement accurately
[70] Unfavorable base stains will not be optimal and thus calculation of the entire loop can be skipped

significant development as Python is notorious for its reliance on indentation[71]. In essence, this will involve adapting PyTMs to the syntax of Python 3 to ensure that it will still work on newer versions of PyMOL. The plugin has already been updated with some minor bug fixes and additional PTMs and is currently at version 1.2.

3.2.3.2 Charge and compatibility with other software

As discussed in Study II the biggest downside is that PyTMs cannot handle charge. This inability is largely inherited from PyMOL itself that has very crude handling of chemistry. However, the flexibility of PyMOL by its integration of Python allows to either import or communicate with 3rd party programs from other sources, e.g. Open Babel [296] or ABPS [297][72], and thus outsource certain functionalities to tools better suited for the task. For instance, there is an existing plugin called 'optimize' for PyMOL using Open Babel to perform structural optimizations within PyMOL.

Accordingly, a future challenge will be to enable compatibility of custom structures with services such as GROMACS [298][73], NAMD [299][74] or AMBER[300][75]. Certainly this may open wider options, e.g. performing molecular dynamics simulation in order to assess the conformation of a protein and using force field services such as AMBER to assess surface electrostatics. However, this adaptation is entirely new territory[76] for myself and adapting compatibility will probably require collaboration to integrate parameters from the other ends. To give an impression, the cost of developing GROMACS alone is estimated to be ~27.5 million US dollars with an effort of over 500 person years and ~1.7 million lines of code.[77] The PyTMs plugin is currently only at 7400 lines of code and less than 2 person years.

3.2.4 CONCLUSIONS AND REFLECTIONS FOR STUDY II

The PyTMs plugin has been extremely useful for myself and others so far, and I hope that development will be continued in some form. However, there are obstacles to overcome if the goal is to implement additional functionality directly, or at least to enable the compatibility to other services. At this point it is a functional and useful explorative tool, provided the limiting caveats are respected. Finally, although the predictive power can be extremely useful in generating hypotheses or explaining observations, ultimately experimental data will be required to fully confirm them.

---

[71] Indentation is critical for correct parsing in Python and enforces a clean style. However most other languages use brackets instead.
[72] http://www.poissonboltzmann.org/
[73] GROMACS: GROningen MAchine for Chemical Simulations; http://www.gromacs.org/
[74] NAMD; http://www.ks.uiuc.edu/Research/namd/
[75] AMBER: Assisted Model Building with Energy Refinement; http://ambermd.org/
[76] One could do a separate PhD education only on molecular dynamics.
[77] http://www.gromacs.org/About_Gromacs

3.3 STUDY III: "NITRATION OF MOG DIMINISHES ITS ENCEPHALITOGENICITY DEPENDING ON MHC HAPLOTYPE"

3.3.1 CONTEXT OF STUDY III

I was intrigued by the fact that MDA-modified MOG increased proliferation of 2D2 cells and demonstrated in Study I that this was attributable to uptake. Importantly I was using PyTMs (Study II) to model various scenarios in relation to MDA, among others the presentation of the MOG_{35-55} epitope by the C57BL/6 MHC molecule H2-IA^b. From that it became apparent that the C-terminal lysine in MOG_{35-55} is expendable for the epitope itself as it far outside of the MHC groove covered by T cell recognition, but that does not exclude potential peripheral interactions. The *in vitro* data however, does not suggest that there are additional effects on antigen presentation, since blocking of uptake reduces the proliferation of 2D2 cell to the same levels as MOG (Study I).

Simultaneously, I was interested in the effect of other modifications on the immunogenicity of MOG. For instance, citrullination of MOG was reported to give rise to cross-creative T cells and thereby contribute to EAE development [301]. This study notably simply used citrullinated MOG_{35-55} peptides to demonstrate cross-reactivity of peptides being citrullinated at R_{41} but not R_{46}. The molecular modeling supported that conclusion, since the R46 is unlikely to accommodate a citrulline at position 46 for appropriate presentation by H2-IA^b and/or T cell recognition. However, molecular modeling of the MOG protein implies that position 46 is more accessible than position 41 (29.7 $Å^2$ solvent-accessible surface area vs. 6.6 $Å^2$). Furthermore, the ability of MOG protein to be citrullinated (especially *in vivo*) is unclear.

At the same time I had established the nitration modification of MOG with the background that protein nitration is a pathological hallmark in MS lesions, but also in conjunction with the C1q project (cf. pg. 20). The initial EAE experiments were performed in DBA1 mice because I was performing experiments related to mainly Study I and I added nitrated MOG as an additional group. These experiments implied that the nitration of MOG would not interfere with its antigenicity. However, it was not until I elucidated the impact of nitration in the 3D model of the MOG_{35-55}/H2-IA^b complex, and performing experiments in C57BL/6 that the protective phenotype became apparent, eventually resulting in Study III.

3.3.2 DISCUSSION OF STUDY III

Study III combines aspects of both Study I and II, namely by being an intersection of biochemistry, immunology and structural bioinformatics. It also is an application example of Study II and unlike Study I there is a robust phenotype that the study builds around.

The immunological implications of antigen nitration in the context of CNS autoimmunity are discussed at length in Study II itself. To summarize, nitration is a mechanism through which T cell responses to specific epitopes can be silenced in conjunction with a specific MHC

haplotype. However, the extent of nitration and its relative contribution to such processes is still elusive.

As not to be redundant of the discussion in the Study itself, in this discussion I will focus more on additional aspects. One notion discussed in the Study is that the degree of nitration *in vivo* remains unclear, and that there are distinctions to be made depending on the source and target of nitration, as well on the immediate tissue-damaging effects and downstream effects on nitrated antigens.

Figure 12: Staining of an EAE spinal cord lesion for nitrotyrosine

This image displays an EAE spinal cord lesion with infiltrate that has been stained with hematoxylin and immunohistochemically for 3-nitrotyrosine (brown color). Protein tyrosine nitration is associated to inflammation.

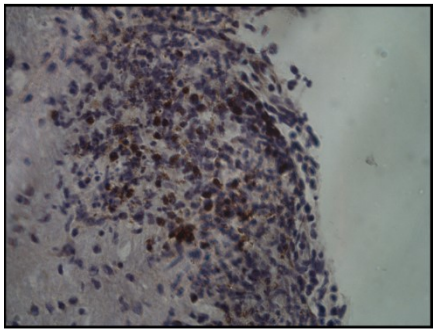
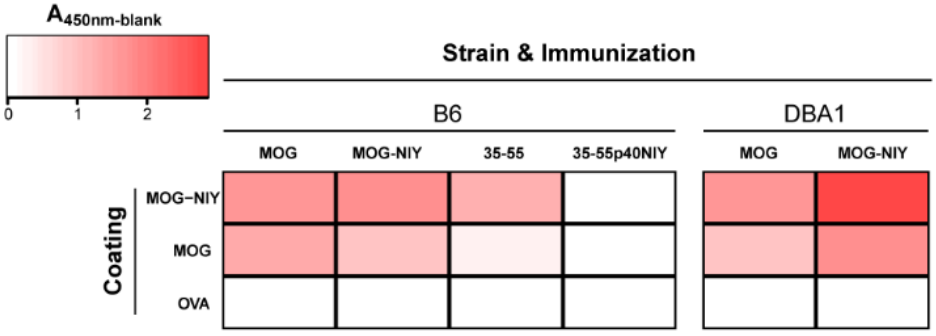


Figure 12 exemplifies that there is abundant nitration within CNS immune infiltrates. Notably, this touches upon the notion that macrophages expose their cargo to oxidative burst involving iNOS and NOX enzymes, typically to kill pathogens. Thus the extent of nitration within cells is possibly modulated by the phagocytosing cell type and its activation state.

Figure 13: Antibody Ig ELISA from immunized mice

The ELISA readout is displayed as a heatmap with red color intensity corresponding to the indicated scale. Each square represents an average value of multiple sera (n=5) taken at the end of EAE experiments.
MOG_{35-55} induces significantly weaker, but detectable antibody responses, whereas $MOG_{35--5p40NIY}$ is negative. Antibodies react with both MOG and MOG-NIY. MOG-NIY immunized mice apparat to have elevated titers toward MOG-NIY, but this may be due to additional NIY-specific antibodies.



Additional data that was not included in Study III itself was serum ELISA from immunized EAE animal (Figure 13). This data confirms the lack of an immune response towards the nitrated APL in C57BL/6 mice. However, there are detectable antibodies towards MOG, even in the MOG-NIY immunized group. This possibly reflects that although the dominant T cell epitope is absent, activation of B cells still is intact, possibly involving hitherto subdominant minor epitopes. These samples were taken at the end of the experiment and therefore most likely reflect secondary responses (especially in the case of peptide immunization). Hence it would be interesting to follow the dynamics of antibody responses over time, and to observe EAE in MOG-NIY immunized mice over longer periods of time.

3.3.3 WHAT ELSE COULD BE DONE?

Although this would be a larger endeavor and has partly been covered by existing studies of pathology, it would be interesting to establish a list of nitrated target proteins in the CNS. We currently know that nitration in the CNS and during inflammation occurs, but there is no immediate evidence that MOG is actually nitrated (nor citrullinated for that matter). One prerequisite would be access to EAE and control tissue, and/or post-mortem lesion and normal-appearing white matter samples. Secondly, a well-designed proteomics work-flow that would enable isolation and analysis of CNS proteins representatively will need be established, especially with regards to the lipid rich brain and membrane proteins, and the low abundance of target candidates such as MOG.

This may also involve specifically labeling nitrations with tags [153] in order to maximize the chances of detection. In Study III the detection was performed label-free, but including labels could further improve the method reliability.

The effects of antigen nitration by various phagocytes should also be addressed. It would be interesting to establish whether specific epitopes become nitrated after uptake, and how the activation sate of the phagocytes factors into that.

An additional aspect of my projects was co-modification, which was never fully explored because the effects of individual modifications were not fully established. First, one would need to work out standard production routines with retained yield as the order of modifications may influence the susceptibility to additional ones (data not included). My prediction is that nitration will exert a *dominant negative effect* over MDA in a 2D2 splenocyte proliferation assay, because it denies the dominant epitope.

Importantly, the non-binding of the nitrated APL should be experimentally confirmed. The lack of direct experimental support currently represents a significant drawback in the consistency of Study III. Although I originally intended to pursue this, the technical issues discussed in Study III currently hamper performing the necessary experiment, I do think they should be performed given the chance. Given how central the MOG_{35-55} epitope and H2-IA^b are to MS

research, and the current development in MHC tetramer technology, I am convinced that the availability of these tools would be an asset in many aspects.

It would be interesting to pursue the question concerning the effect of MOG nitration in MHC congenic strains and hybrids. My expectation would be that the phenotype of $H2^q$ congenic C57BL/6 mice might mimic that of $H2^q$ DBA1 mice. Conversely, $H2^b$ congenic DBA1 mice might resemble the C57BL/6 phenotype. Hybrids possibly retain susceptibility, but it would be interesting to determine whether the immune response towards nitrated MOG is initiated first against the MOG_{79-98} epitope and then spreads to MOG_{35-55} from endogenous MOG.

Lastly, the remaining issue is the clinical translation into MS. As discussed in the study itself, there are considerable differences to the heterogeneous epitope responses in MS patients. Defining the epitope specificities of MS HLA risk haplotypes has been a challenge in the field. Future studies and approaches will hopefully identify defined complexes that will give more insight into the autoantigens in MS and their presentation.

3.3.4 CONCLUDING REFLECTIONS ON STUDY III

Overall this study is a nice example of the applicability of Study II and antigen nitration as a mechanism that regulates susceptibility to autoimmune CNS inflammation and dependence on genetic background. Although Study III is an independent story, experimental confirmation of the lack of binding for the nitrated APL predicted by bioinformatics would be highly desirable. Lastly, there are many implications that remain to be explored, as well as related open questions that await clarification.

3.4 STUDY IV: "RAT BONE MARROW-DERIVED DENDRITIC CELLS GENERATED WITH GM-CSF/IL-4 OR FLT3L EXHIBIT DISTINCT PHENOTYPICAL AND FUNCTIONAL CHARACTERISTICS"

3.4.1 HISTORIC CONTEXT OF STUDY IV

The entry point into this study was from a very different angle. My fellow PhD colleague Marie, who is the first author, was characterizing these cells in the rat in relation to her projects dedicated to deciphering the role of C-type lectins genetically associated to EAE susceptibility.

My angle was an entirely different one. Based on the prevailing literature at the time, I was still hoping to observe strong pro-inflammatory activation of various APCs in response to MOG-MDA (Study I) and to decipher the mechanism behind it. Having bone-marrow-derived dendritic cells (BMDCs) available would greatly enable studying different populations *in vitro*. Secondly, I was questioning the active EAE model with the dilemma that the CFA was potentially overriding delicate alterations of immune responses owing to PTMs. A passive model of EAE that is based on BMDCs rather than T cells could thus potentially be an option to explore and functionally address the role of APCs in EAE. Later it became apparent though that classical DCs do not express SRA and thus do not have the capacity to take up MOG-MDA selectively. Besides we have thus far not succeeded to induce EAE symptoms by transfer of antigen-loaded BMDCs.

3.4.2 DISCUSSION OF STUDY IV

While I was involved in the project we conducted a series of pilot experiments trying to observe immune responses towards antigen after transfer into naïve rats. These experiments were largely inconclusive and are not part of Study IV. A major problem is that we failed to observe antigen-specific responses[78] to either MBP or MOG when assaying the lymph nodes of rats following transfer, nor did they develop EAE. We did, however, gather evidence that transferred FLT3L BMDCs migrated to lymph nodes. Possibly the use of a foreign as opposed to a self-antigen might be an approach. Alternatively, a bystander immunization may be required to evoke lasting stimulation. However, several challenges related to cell viability, purity and antigen responses still remain.

A second major obstacle that is discussed in Study IV is the heterogeneity of cell populations within the respective protocols, i.e. FLT3L or GM-CSF/IL-4. In retrospect we could have tried to account for these differences and/or tried to optimize culture condition to passively select homogenous cells. Presently this is still not quite feasible. First it needs to be elucidated which additional factors could potentially be applied. The characterized BMDCs as such are nonetheless still invaluable tools for *in vitro* assays.

[78] The responses were elevated but appeared to respond to culture media. Attempts to culture cells in serum-free or rat serum containing media were undertaken but met technical limits.

4 CONCLUDING REFLECTIONS AND FUTURE PERSPECTIVES

The projects within this thesis relate to the role of post-translational modifications in CNS autoimmunity and inflammation. The overarching aim is to elucidate mechanisms of immunology and to improve the understanding of complex processes within diseases such as MS, and lastly to identify leads for therapeutic intervention.

Study I has been at the heart of the project and was a valuable learning experience, although overall it did not follow the plan intended for me. The scientific aspects aside, this project was defining for my development as a scientist. Personally it highlighted several aspects of scientific philosophy: thorough and self-critical methodology yielding conclusive and reliable results, critical interpretation of the literature, scientific reading in general to expand the horizon and the importance of good project design with either a fail proof hypothesis–generating approach or a solid phenotype at heart.

As far as the science is concerned Study I confirms in an elegant way that the lipid peroxidation end products of MDA render the carrier protein into a ligand for SRA. The association of SRA expression and increased uptake to homeostatic M2-type macrophages is a novel aspect implying a more regulated response to MDA adducts that have been demonized in the context of autoimmunity. I voiced skepticism towards default pro-inflammatory effects of MDA adducts, which I believe are instead attributable to soluble MDA. Dissecting their individual effects would be valuable to understanding their impact and risk for autoimmunity, especially since lipid peroxidation and MDA adduct formation are not necessarily dissociated *in vivo*. Understanding these mechanisms and their individual impact on immunology will be critical to draw a more diversified picture of the role of PTMs in autoimmunity.

Study II and the development of the PyTMs plugin had its roots in the infeasibility of pursuing crystallization of MOG-MDA. However, although initially being a side-track, I believe that due to the considerable investments that were made in its development, the plugin can potnetially contribute more to the research of PTMs in the long run. There are some limiting caveats and challenges associated to its future development, however, this could be a slowly evolving project that will require collaboration.

Notably, **Study III** is already an application example of the PyTMs plugin allowing me to address research questions and to explain experimental observations at a molecular level. The design of this project was significantly more straightforward and based on a clear phenotype that facilitated the process. Although experimental validation for the conclusion that presentation by H2-IA^{b} is indeed denied is pending, I consider the likelihood of confirmation is theoretically well supported. Nonetheless, I fully agree with the notion that experimental evidence would significantly bolster the conclusion and tie up the story. Apart from that, having H2-IA^{b} complexes available for other assays (e.g. tetramer staining of MOG_{35-55} reactive T cells) would be invaluable tools for other projects.

The observation that nitration diminishes immune responses to specific epitopes depending on the MHC background is extremely intriguing in the context of risk haplotypes and genetic predisposition, but also has implications on the dynamics of immune responses. Additional examples and the relative contribution to shaping immune response *in vivo* remain to be investigated.

Although from its content **Study IV** is not immediately connected to the theme of PTMs the intent with participation from my side was to develop new methods and tools to address immunological questions related to modified antigen. Similar to Study II, Study IV is indicative of a quest for new techniques and tools to enable scientists to address questions with a novel approach. Eventually the application of new technologies is a key factor driving scientific progress. Our thorough characterization in Study IV reflects that establishing, optimizing and defining methods and tools is something that it integral to scientific progress.

This thesis is very diverse in the ground that it covers. I have built up many aspects of the projects from scratch, starting from bacteria to produce my own protein material, chemical modification and fluorescent labeling, biochemical analyses and mass spectrometry all the way to *in vivo* applications. This was paralleled by bioinformatics approaches, molecular modeling and modern data analyses. Altogether these provide a valuable skill set for quality science.

In summary, the studies and this thesis provide both tools and more diversified interpretations in the context of post-translational modifications in autoimmunity, particularly for myelin oligodendrocyte glycoprotein and CNS autoimmunity. The interactions in immunology are extremely complex. Thus every individual study represents a small piece of a gigantic evolving puzzle that is just beginning to outline a fascinating and enigmatic picture that we cannot begin to fully grasp quite yet.

5 ACKNOWLEDGEMENTS

It was a pleasure to work surrounded by so many brilliant and hard-working people which I would like to thank individually below. Before I do so, I generally thank **Karolinska Institutet**, the **Department of Clinical Neuroscience** and the **Center for Molecular Medicine** for support and providing the foundations for conducting research. I also acknowledge the efforts of administrative and maintenance staff that keep everything running.

I want to start by expressing my gratitude to **Robert 'Bob' Harris**. When I started in your lab you gifted me a copy of '*The Old Man and the Sea*' by Ernest Hemingway with the remark: "It's about not giving up". Naive as I was, I figured this reminder wouldn't apply to me because, 'naturally', the road to my PhD would be a walk in the park. In reality my PhD ended up having many parallels to the novel, but I learned valuable life lessons that science alone could not teach me. You gave me freedom to develop as a scientist and a person. The discussions with you have been inspiring and insightful and I value your experience. Thank you for giving me this opportunity!

I am also grateful to my co-supervisors **Jonas Bergquist** and **Adnane Achour** for all your support and valuable feedback. Your individual expertise was instrumental to my different projects and I have learned a great deal from interacting with you. It was a pleasure to perform and discuss research with you.

I am indebted to **Tomas Olsson** who is a true patron and inspiration to us all. You embody the spirit of our Neuroimmunology Unit and foster a unique and international environment. I cherish your obligatory contribution to entertainment at dissertation dinners and our time in *Pärlbandet*.

It was a privilege to be surrounded by inspiring group leaders within the lab: **Maja Jagodic**, **Fredrik Piehl, Ingrid Kockum** and **Lou Brundin**. You are all role models for upcoming scientist in your unique way and I appreciate your professional work.

I thank everyone from the **Neuroimmunology Unit** and our **Rheumatology** neighbors.

Much credit has to be given to my master student **Sonja**. You have worked exceptionally hard during your time in the lab and earned your rewards. It was an inspiration to work with you and this period became the most enthusiastic and productive time of my PhD. You were a true catalyst and the cure for procrastination. Without your help I would have been unable to perform several key experiments. I genuinely cherish the time you were here.

Marie, the sister I never had, and needed! You've influenced me positively in so many ways: from pimping my wardrobe to having a positive perspective and teaching me to dance Lindy Hop. I admire your energy and attitude, there is no party without you. There are so many anecdotes that come to mind throughout the years. It was a privilege to work with you!

Adam & Erika. If it wasn't for your support I would not be where I am right now. You've welcomed me and helped me countless times since our undergraduate studies, especially in difficult times. I truly appreciate your support and encouragement!

Also the rest of the gang: **Gustav & Åsa**, **Espen**, **Avi**, **Ron R. & Sarit**, **Ron A. & Nok**, **Taz & Ammara** and **Deborah**. Thank you for all the good times. Some of my most treasurable memories are thanks to you all!

I want to thank my colleagues, collaborators and people from KI:

Anatoly, for discussion on protein purification. **André**, for inspiration, pragmatism and support. You are a role model for post docs. **Brinda**, for your help with ordering and the chemical room. **Cynthia**, good luck with your postdoc. **Eliane**, for feedback and injecting life to the CMM pub. **Hannes**, for being such a nice officemate. **Harald**, for your help with immunohistochemistry and the confocal. **Izaura**, for assistance with the Biomek. **Jonas**, for enjoyable conversations in L5. **Kelly**, good luck on your way! **Lara** 'la petit femme', for company and casual conversations. **Lasse**, for introducing me to the ÄKTA. **Matthias**, for your positive feedback, assistance and conversations. Good luck with your residency. **Melanie P.**, for taking over responsibility for the chemical room (*evil laugh* ☺). Good luck with your projects. **Mohsen**, the master of logistics and procurement, the man standing between the lab and total chaos. Thank you for your assistance with everything and looking out for me. **Nada**, for your kindness and generosity, I respect that immensely. **Pernilla**, for advice on statistics and your pragmatism. **Roham**, for introducing me to Readcube. **Sahl**, thanks for being around and good conversations. **Sabrina**, for multiple occasions helping out, also for selfless assistance with the inventories. **Sevi**, for moral support during writing the thesis! Είμαι

κουρασμένος. Θέλω καφέ, θέλω διακοπές! **Shahin**, the office it not the same without your spontaneous raps. I admire your general knowledge and technical expertise. **Sohel**, it was my pleasure to work with you and I cherish our discussions. **Sravani**, for all your help with the Mass Spectrometry and analysis. **Susi**, you have amazing positive energy, it's good to have people like you in the lab. **Tatiana**, for coffee breaks and dances at Chicago! **Tatyana**, for introducing me to PyMOL. **Ulrika Olsson**, for making KI a safer working place and introducing barcodes. **Uta**, for discussions on MDA. **Venus**, the MOG queen! You've helped me considerably throughout the years and do so many integral things to the lab in the background. Thank you for all the encouragement and support and the most amazing cakes! **Xingmei**, for bringing joyful energy to the group and unusually entertaining stories.

My appreciation to all **rheumatology technicians** for supporting me with the chemical room.

Additional thanks to:

Present and previous people from the corridor: **Alexandra**, **Agnes**, **Cecilia**, **Ewoud**, **Faiez**, **Galina**, **Hannah**, **Karl**, **Magda**, **Magnus**, **Manuel & Milena**, **Melanie**, **Mikael**, **Nånnis**, **Patrick**, **Petra**, **Rasmus**, **Rickard**, **Rux**, **Sreeni** and **Tojo**.

Stephan Meinke and **Petter Höglund** for forthcoming assistance with the C1q project. It was worth a try. **Elisabeth** and **Sandra** representatively for all the **AKM staff** and **Elin** representatively for the **CNS administration** for professional support. **Dan Grandér**, **Jonas Sundbäck** and **Elias Arnér** for your support in my undergraduate times.

Thanks to everyone that helped to feed the fish. Apologies to anyone I may have overlooked.

Miriam & Janne, thank you for your support. An exciting life lies ahead of you and I wish you good luck. **Fedor Nikulenkov**, you have inspired me early on to strive for excellent science. Also thanks to **Clemens Spinnler**, I totally understand you now. Thank you both for beers and ice hockey! **Mike** representatively for Project Sentinel, thanks for many epic moments. A big shout-out to the **PyMOL community** and wiki contributors. Thanks to **Thomas Holder** for support and feedback.

Jag vill även tacka de som har välkomnat mig som en ny familjemedlem:

Britta & Kent, för alla trevliga kvällar och god mat vid våra utflykter till stugan. **Ulla & Roland**, **Daniel**, **Hans-Erik** och **Bea**, för trevliga besök här i Stockholm och hemma hos er i Dalbyn.

Ich möchte vor allem meiner Familie danken, auf die ich mich in allen Lebenslagen verlassen kann. Ich bin froh, dass es euch gibt und wäre nicht derselbe ohne euch. Durch den räumlichen Abstand habe ich in den letzten Jahren einiges verpasst, aber ihr seid mir immer nahe am Herzen. Wenn wir uns alle sehen ist es immer umso schöner. Danke für alles was uns verbindet, Worte können das nicht zum Ausdruck bringen.

Rosi & Peter: Ihr wart immer für mich da und habt einiges auf euch genommen um uns in der Kindheit zu begleiten - das ist nicht selbstverständlich. Ich freue mich immer euch zu besuchen und über die Anrufe.

Margitta & Werner: Ihr habt mich in allem gefördert und mir alles ermöglicht. Ich verdanke euch alles.

Marc, Ralf & Frank: die wilden Jahre sind glücklicherweise lange vorbei ☺ Jetzt da wir alle erwachsen sind ist es toll, dass wir uns alle so gut verstehen!

Alina & Rosa: Ihr seid beide so herzliche Menschen und es freut mich wie ihr die Familie ergänzt.

Sara. Jag vet inte hur jag skulle kunna tacka dig för all ditt stöd. Jag skulle nog inte ha klarat mig om det inte ha varit för din skull. Nära dig är jag mig själv och du kallar fram det bästa i mig. Jag älskar dig och alla skratten vi delar.

6 REFERENCES

1. Dendrou, C.A., L. Fugger, and M.A. Friese, *Immunopathology of multiple sclerosis.* Nat Rev Immunol, 2015. **15**(9): p. 545-58.
2. Harbo, H.F., R. Gold, and M. Tintoré, *Sex and gender issues in multiple sclerosis.* Ther Adv Neurol Disord, 2013. **6**(4): p. 237-48.
3. Olsson, T., *The new era of multiple sclerosis therapy.* J Intern Med, 2014. **275**(4): p. 382-6.
4. Landtblom, A.M., et al., *Distribution of multiple sclerosis in Sweden based on mortality and disability compensation statistics.* Neuroepidemiology, 2002. **21**(4): p. 167-79.
5. Ahlgren, C., A. Odén, and J. Lycke, *High nationwide prevalence of multiple sclerosis in Sweden.* Mult Scler, 2011. **17**(8): p. 901-8.
6. Kurtzke, J.F., *Historical and clinical perspectives of the expanded disability status scale.* Neuroepidemiology, 2008. **31**(1): p. 1-9.
7. McCarthy, C. and J. Thorpe, *Some recent advances in multiple sclerosis.* J Neurol, 2016.
8. Simons, M. and K.A. Nave, *Oligodendrocytes: Myelination and Axonal Support.* Cold Spring Harb Perspect Biol, 2016. **8**(1): p. a020479.
9. Sherman, D.L. and P.J. Brophy, *Mechanisms of axon ensheathment and myelin growth.* Nat Rev Neurosci, 2005. **6**(9): p. 683-90.
10. Hughes, E.G. and B. Appel, *The cell biology of CNS myelination.* Curr Opin Neurobiol, 2016. **39**: p. 93-100.
11. Lassmann, H., J. van Horssen, and D. Mahad, *Progressive multiple sclerosis: pathology and pathogenesis.* Nat Rev Neurol, 2012. **8**(11): p. 647-56.
12. Steinman, L., *Immunology of relapse and remission in multiple sclerosis.* Annu Rev Immunol, 2014. **32**: p. 257-81.
13. Nikić, I., et al., *A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis.* Nat Med, 2011. **17**(4): p. 495-9.
14. Mkhikian, H., et al., *Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis.* Nat Commun, 2011. **2**: p. 334.
15. Ascherio, A., K.L. Munger, and J.D. Lünemann, *The initiation and prevention of multiple sclerosis.* Nat Rev Neurol, 2012. **8**(11): p. 602-12.
16. Martino, G. and H.P. Hartung, *Immunopathogenesis of multiple sclerosis: the role of T cells.* Curr Opin Neurol, 1999. **12**(3): p. 309-21.
17. Piehl, F., *A changing treatment landscape for multiple sclerosis: challenges and opportunities.* J Intern Med, 2014. **275**(4): p. 364-81.
18. Goverman, J.M., *Immune tolerance in multiple sclerosis.* Immunol Rev, 2011. **241**(1): p. 228-40.
19. Rangachari, M. and V.K. Kuchroo, *Using EAE to better understand principles of immune function and autoimmune pathology.* J Autoimmun, 2013. **45**: p. 31-9.
20. Sie, C., T. Korn, and M. Mitsdoerffer, *Th17 cells in central nervous system autoimmunity.* Exp Neurol, 2014. **262 Pt A**: p. 18-27.
21. Chastain, E.M., et al., *The role of antigen presenting cells in multiple sclerosis.* Biochim Biophys Acta, 2011. **1812**(2): p. 265-74.
22. Stoeckle, C. and E. Tolosa, *Antigen processing and presentation in multiple sclerosis.* Results Probl Cell Differ, 2010. **51**: p. 149-72.
23. Odoardi, F., et al., *T cells become licensed in the lung to enter the central nervous system.* Nature, 2012. **488**(7413): p. 675-9.
24. Croxford, A.L., et al., *The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2+ Monocytes and Licenses Autoimmunity.* Immunity, 2015. **43**(3): p. 502-14.
25. Codarri, L., et al., *RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation.* Nat Immunol, 2011. **12**(6): p. 560-7.
26. Yamasaki, R., et al., *Differential roles of microglia and monocytes in the inflamed central nervous system.* J Exp Med, 2014. **211**(8): p. 1533-49.
27. Simmons, S.B., et al., *Modeling the heterogeneity of multiple sclerosis in animals.* Trends Immunol, 2013. **34**(8): p. 410-22.
28. Tuohy, V.K., et al., *The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis.* Immunol Rev, 1998. **164**: p. 93-100.
29. Tuohy, V.K., et al., *Regression and spreading of self-recognition during the development of autoimmune demyelinating disease.* J Autoimmun, 1999. **13**(1): p. 11-20.
30. Krumbholz, M., et al., *B cells and antibodies in multiple sclerosis pathogenesis and therapy.* Nat Rev Neurol, 2012. **8**(11): p. 613-23.
31. Barr, T.A., et al., *B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells.* J Exp Med, 2012. **209**(5): p. 1001-10.

32. Molnarfi, N., et al., *MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies.* J Exp Med, 2013. **210**(13): p. 2921-37.
33. Mayer, M.C. and E. Meinl, *Glycoproteins as targets of autoantibodies in CNS inflammation: MOG and more.* Ther Adv Neurol Disord, 2012. **5**(3): p. 147-59.
34. Flytzani, S., et al., *MOG-induced experimental autoimmune encephalomyelitis in the rat species triggers anti-neurofascin antibody response that is genetically regulated.* J Neuroinflammation, 2015. **12**: p. 194.
35. Schirmer, L., et al., *Differential loss of KIR4.1 immunoreactivity in multiple sclerosis lesions.* Ann Neurol, 2014. **75**(6): p. 810-28.
36. Johns, T.G. and C.C. Bernard, *The structure and function of myelin oligodendrocyte glycoprotein.* J Neurochem, 1999. **72**(1): p. 1-9.
37. Reindl, M., et al., *The spectrum of MOG autoantibody-associated demyelinating diseases.* Nat Rev Neurol, 2013. **9**(8): p. 455-61.
38. Elliott, C., et al., *Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis.* Brain, 2012. **135**(Pt 6): p. 1819-33.
39. Mayer, M.C., et al., *Distinction and temporal stability of conformational epitopes on myelin oligodendrocyte glycoprotein recognized by patients with different inflammatory central nervous system diseases.* J Immunol, 2013. **191**(7): p. 3594-604.
40. Gaertner, S., et al., *Antibodies against glycosylated native MOG are elevated in patients with multiple sclerosis.* Neurology, 2004. **63**(12): p. 2381-3.
41. Menge, T., et al., *Conformational epitopes of myelin oligodendrocyte glycoprotein are targets of potentially pathogenic antibody responses in multiple sclerosis.* J Neuroinflammation, 2011. **8**: p. 161.
42. Ramanathan, S., R.C. Dale, and F. Brilot, *Anti-MOG antibody: The history, clinical phenotype, and pathogenicity of a serum biomarker for demyelination.* Autoimmun Rev, 2016. **15**(4): p. 307-24.
43. Srivastava, R., et al., *Potassium channel KIR4.1 as an immune target in multiple sclerosis.* N Engl J Med, 2012. **367**(2): p. 115-23.
44. Pröbstel, A.K., et al., *Multiple Sclerosis and Antibodies against KIR4.1.* N Engl J Med, 2016. **374**(15): p. 1496-8.
45. Kraus, V., et al., *Potassium channel KIR4.1-specific antibodies in children with acquired demyelinating CNS disease.* Neurology, 2014. **82**(6): p. 470-3.
46. Chastre, A., D.A. Hafler, and K.C. O'Connor, *Evaluation of KIR4.1 as an Immune Target in Multiple Sclerosis.* N Engl J Med, 2016. **374**(15): p. 1495-6.
47. Filippi, M., M.A. Rocca, and H. Lassmann, *KIR4.1: another misleading expectation in multiple sclerosis?* Lancet Neurol, 2014. **13**(8): p. 753-5.
48. Herz, J., F. Zipp, and V. Siffrin, *Neurodegeneration in autoimmune CNS inflammation.* Exp Neurol, 2010. **225**(1): p. 9-17.
49. Mahad, D., H. Lassmann, and D. Turnbull, *Review: Mitochondria and disease progression in multiple sclerosis.* Neuropathol Appl Neurobiol, 2008. **34**(6): p. 577-89.
50. Lassmann, H., W. Brück, and C. Lucchinetti, *Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy.* Trends Mol Med, 2001. **7**(3): p. 115-21.
51. Moutsianas, L., et al., *Class II HLA interactions modulate genetic risk for multiple sclerosis.* Nat Genet, 2015. **47**(10): p. 1107-13.
52. Scally, S.W., et al., *A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis.* J Exp Med, 2013. **210**(12): p. 2569-82.
53. Piehl, F. and T. Olsson, *Inflammation and susceptibility to neurodegeneration: the use of unbiased genetics to decipher critical regulatory pathways.* Neuroscience, 2009. **158**(3): p. 1143-50.
54. Guerreiro-Cacais, A.O., et al., *Translational utility of experimental autoimmune encephalomyelitis: recent developments.* J Inflamm Res, 2015. **8**: p. 211-25.
55. Axisa, P.P. and D.A. Hafler, *Multiple sclerosis: genetics, biomarkers, treatments.* Curr Opin Neurol, 2016. **29**(3): p. 345-53.
56. Perricone, C., et al., *Smoke and autoimmunity: The fire behind the disease.* Autoimmun Rev, 2016. **15**(4): p. 354-74.
57. Sundqvist, E., et al., *Epstein-Barr virus and multiple sclerosis: interaction with HLA.* Genes Immun, 2012. **13**(1): p. 14-20.
58. Hedström, A.K., T. Olsson, and L. Alfredsson, *High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women.* Mult Scler, 2012. **18**(9): p. 1334-6.
59. Hedström, A.K., et al., *Shift work at young age is associated with increased risk for multiple sclerosis.* Ann Neurol, 2011. **70**(5): p. 733-41.
60. Breithaupt, C., et al., *Structural insights into the antigenicity of myelin oligodendrocyte glycoprotein.* Proc Natl Acad Sci U S A, 2003. **100**(16): p. 9446-51.
61. VanAmerongen, B.M., et al., *Multiple sclerosis and vitamin D: an update.* Eur J Clin Nutr, 2004. **58**(8): p. 1095-109.

62. Fernandes de Abreu, D.A., V. Landel, and F. Féron, *Seasonal, gestational and postnatal influences on multiple sclerosis: the beneficial role of a vitamin D supplementation during early life.* J Neurol Sci, 2011. **311**(1-2): p. 64-8.
63. Adzemovic, M.Z., et al., *Efficacy of vitamin D in treating multiple sclerosis-like neuroinflammation depends on developmental stage.* Exp Neurol, 2013. **249**: p. 39-48.
64. Joshi, S., et al., *1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A.* Mol Cell Biol, 2011. **31**(17): p. 3653-69.
65. Burkett, P.R., G. Meyer zu Horste, and V.K. Kuchroo, *Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity.* J Clin Invest, 2015. **125**(6): p. 2211-9.
66. DeLuca, H.F. and L.A. Plum, *Vitamin D deficiency diminishes the severity and delays onset of experimental autoimmune encephalomyelitis.* Arch Biochem Biophys, 2011. **513**(2): p. 140-3.
67. Dopico, X.C., et al., *Widespread seasonal gene expression reveals annual differences in human immunity and physiology.* Nat Commun, 2015. **6**: p. 7000.
68. Benson, C., et al., *Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE).* Exp Neurol, 2015. **271**: p. 279-90.
69. Hart, P.H., S. Gorman, and J.J. Finlay-Jones, *Modulation of the immune system by UV radiation: more than just the effects of vitamin D?* Nat Rev Immunol, 2011. **11**(9): p. 584-96.
70. Hedström, A.K., et al., *Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis.* Neurology, 2009. **73**(9): p. 696-701.
71. Hedström, A.K., et al., *Nicotine might have a protective effect in the etiology of multiple sclerosis.* Mult Scler, 2013. **19**(8): p. 1009-13.
72. Hedström, A.K., et al., *Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis.* Brain, 2011. **134**(Pt 3): p. 653-64.
73. Calabresi, P.A., et al., *Pegylated interferon β-1a for relapsing-remitting multiple sclerosis (ADVANCE): a randomised, phase 3, double-blind study.* Lancet Neurol, 2014. **13**(7): p. 657-65.
74. Kieseier, B.C., *The mechanism of action of interferon-β in relapsing multiple sclerosis.* CNS Drugs, 2011. **25**(6): p. 491-502.
75. Durelli, L., et al., *T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta.* Ann Neurol, 2009. **65**(5): p. 499-509.
76. Lee, L.F., et al., *IL-7 promotes T(H)1 development and serum IL-7 predicts clinical response to interferon-β in multiple sclerosis.* Sci Transl Med, 2011. **3**(93): p. 93ra68.
77. Fox, R.J., et al., *Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis.* N Engl J Med, 2012. **367**(12): p. 1087-97.
78. Gold, R., et al., *Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis.* N Engl J Med, 2012. **367**(12): p. 1098-107.
79. Tang, T. and A. Takasu, *Facile synthesis of unsaturated polyester-based double-network gels via chemoselective cross-linking using Michael addition and subsequent UV-initiated radical polymerization.* RSC Advances, 2015. **5**(2): p. 819-829.
80. Ropper, A.H., *The "poison chair" treatment for multiple sclerosis.* N Engl J Med, 2012. **367**(12): p. 1149-50.
81. Rantanen, T., *The cause of the Chinese sofa/chair dermatitis epidemic is likely to be contact allergy to dimethylfumarate, a novel potent contact sensitizer.* Br J Dermatol, 2008. **159**(1): p. 218-21.
82. Franklin, R.J., et al., *Neuroprotection and repair in multiple sclerosis.* Nat Rev Neurol, 2012. **8**(11): p. 624-34.
83. Pham-Dinh, D., et al., *Structure of the human myelin/oligodendrocyte glycoprotein gene and multiple alternative spliced isoforms.* Genomics, 1995. **29**(2): p. 345-52.
84. Modrek, B. and C.J. Lee, *Alternative splicing in the human, mouse and rat genomes is associated with an increased frequency of exon creation and/or loss.* Nat Genet, 2003. **34**(2): p. 177-80.
85. Delarasse, C., et al., *Complex alternative splicing of the myelin oligodendrocyte glycoprotein gene is unique to human and non-human primates.* J Neurochem, 2006. **98**(6): p. 1707-17.
86. Evsyukova, I., et al., *Alternative splicing in multiple sclerosis and other autoimmune diseases.* RNA Biol, 2010. **7**(4): p. 462-73.
87. Kent, W.J., et al., *The human genome browser at UCSC.* Genome Res, 2002. **12**(6): p. 996-1006.
88. Boyle, L.H., et al., *Splice variation in the cytoplasmic domains of myelin oligodendrocyte glycoprotein affects its cellular localisation and transport.* J Neurochem, 2007. **102**(6): p. 1853-62.
89. Linnington, C., M. Webb, and P.L. Woodhams, *A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody.* J Neuroimmunol, 1984. **6**(6): p. 387-96.
90. Clements, C.S., et al., *The crystal structure of myelin oligodendrocyte glycoprotein, a key autoantigen in multiple sclerosis.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 11059-64.
91. Johns, T.G. and C.C. Bernard, *Binding of complement component Clq to myelin oligodendrocyte glycoprotein: a novel mechanism for regulating CNS inflammation.* Mol Immunol, 1997. **34**(1): p. 33-8.
92. von Büdingen, H.C., et al., *The myelin oligodendrocyte glycoprotein directly binds nerve growth factor to modulate central axon circuitry.* J Cell Biol, 2015. **210**(6): p. 891-8.

93. Smith, I.A., et al., *BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation.* J Immunol, 2010. **184**(7): p. 3514-25.
94. Palakodeti, A., et al., *The molecular basis for modulation of human Vγ9Vδ2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies.* J Biol Chem, 2012. **287**(39): p. 32780-90.
95. Blink, S.E. and S.D. Miller, *The contribution of gammadelta T cells to the pathogenesis of EAE and MS.* Curr Mol Med, 2009. **9**(1): p. 15-22.
96. Stefferl, A., et al., *Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis.* J Immunol, 2000. **165**(5): p. 2859-65.
97. Guggenmos, J., et al., *Antibody cross-reactivity between myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis.* J Immunol, 2004. **172**(1): p. 661-8.
98. Delarasse, C., et al., *Myelin/oligodendrocyte glycoprotein-deficient (MOG-deficient) mice reveal lack of immune tolerance to MOG in wild-type mice.* J Clin Invest, 2003. **112**(4): p. 544-53.
99. Hundgeburth, L.C., et al., *The complement system contributes to the pathology of experimental autoimmune encephalomyelitis by triggering demyelination and modifying the antigen-specific T and B cell response.* Clin Immunol, 2013. **146**(3): p. 155-64.
100. Ramaglia, V., et al., *C3-dependent mechanism of microglial priming relevant to multiple sclerosis.* Proc Natl Acad Sci U S A, 2012. **109**(3): p. 965-70.
101. Nayak, A., et al., *The non-classical functions of the classical complement pathway recognition subcomponent C1q.* Immunol Lett, 2010. **131**(2): p. 139-50.
102. Ghebrehiwet, B., et al., *The C1q family of proteins: insights into the emerging non-traditional functions.* Front Immunol, 2012. **3**.
103. Naito, A.T., et al., *Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes.* Cell, 2012. **149**(6): p. 1298-313.
104. Weismann, D., et al., *Complement factor H binds malondialdehyde epitopes and protects from oxidative stress.* Nature, 2011. **478**(7367): p. 76-81.
105. Veneskoski, M., et al., *Specific recognition of malondialdehyde and malondialdehyde acetaldehyde adducts on oxidized LDL and apoptotic cells by complement anaphylatoxin C3a.* Free Radic Biol Med, 2011. **51**(4): p. 834-43.
106. García-Vallejo, J.J., et al., *CNS myelin induces regulatory functions of DC-SIGN-expressing, antigen-presenting cells via cognate interaction with MOG.* J Exp Med, 2014. **211**(7): p. 1465-83.
107. Hor, H., et al., *A missense mutation in myelin oligodendrocyte glycoprotein as a cause of familial narcolepsy with cataplexy.* Am J Hum Genet, 2011. **89**(3): p. 474-9.
108. Ahmed, S.S. and L. Steinman, *Mechanistic insights into influenza vaccine-associated narcolepsy.* Hum Vaccin Immunother, 2016: p. 1-6.
109. Bernard, C.C., et al., *Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis.* J Mol Med (Berl), 1997. **75**(2): p. 77-88.
110. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun, *A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells.* Eur J Immunol, 1995. **25**(7): p. 1951-9.
111. Petersen, T.R., et al., *Characterization of MHC- and TCR-binding residues of the myelin oligodendrocyte glycoprotein 38-51 peptide.* Eur J Immunol, 2004. **34**(1): p. 165-73.
112. Sweenie, C.H., et al., *Distinct T cell recognition of naturally processed and cryptic epitopes within the immunodominant 35-55 region of myelin oligodendrocyte glycoprotein.* J Neuroimmunol, 2007. **183**(1-2): p. 7-16.
113. Ben-Nun, A., et al., *Anatomy of T cell autoimmunity to myelin oligodendrocyte glycoprotein (MOG): prime role of MOG44F in selection and control of MOG-reactive T cells in H-2b mice.* Eur J Immunol, 2006. **36**(2): p. 478-93.
114. Shetty, A., et al., *Immunodominant T-cell epitopes of MOG reside in its transmembrane and cytoplasmic domains in EAE.* Neurol Neuroimmunol Neuroinflamm, 2014. **1**(2): p. e22.
115. Abdul-Majid, K.B., et al., *Screening of several H-2 congenic mouse strains identified H-2(q) mice as highly susceptible to MOG-induced EAE with minimal adjuvant requirement.* J Neuroimmunol, 2000. **111**(1-2): p. 23-33.
116. Wallström, E., et al., *Increased reactivity to myelin oligodendrocyte glycoprotein peptides and epitope mapping in HLA DR2(15)+ multiple sclerosis.* Eur J Immunol, 1998. **28**(10): p. 3329-35.
117. Koehler, N.K., et al., *The human T cell response to myelin oligodendrocyte glycoprotein: a multiple sclerosis family-based study.* J Immunol, 2002. **168**(11): p. 5920-7.
118. Breithaupt, C., et al., *Demyelinating myelin oligodendrocyte glycoprotein-specific autoantibody response is focused on one dominant conformational epitope region in rodents.* J Immunol, 2008. **181**(2): p. 1255-63.
119. Bergman, C.M., et al., *A switch in pathogenic mechanism in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in IFN-γ-inducible lysosomal thiol reductase-free mice.* J Immunol, 2012. **188**(12): p. 6001-9.

120. Cong, H., Y. Jiang, and P. Tien, *Identification of the myelin oligodendrocyte glycoprotein as a cellular receptor for rubella virus.* J Virol, 2011. **85**(21): p. 11038-47.
121. *Rubella vaccines: WHO position paper.* Wkly Epidemiol Rec, 2011. **86**(29): p. 301-16.
122. Oliver, A.R., G.M. Lyon, and N.H. Ruddle, *Rat and human myelin oligodendrocyte glycoproteins induce experimental autoimmune encephalomyelitis by different mechanisms in C57BL/6 mice.* J Immunol, 2003. **171**(1): p. 462-8.
123. Gold, R., C. Linington, and H. Lassmann, *Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research.* Brain, 2006. **129**(Pt 8): p. 1953-71.
124. McCarthy, D.P., M.H. Richards, and S.D. Miller, *Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease.* Methods Mol Biol, 2012. **900**: p. 381-401.
125. Greve, B., et al., *Dissimilar background genes control susceptibility to autoimmune disease in the context of different MHC haplotypes: NOD.H-2(s) congenic mice are relatively resistant to both experimental autoimmune encephalomyelitis and type I diabetes.* Eur J Immunol, 2004. **34**(7): p. 1828-38.
126. Flytzani, S., et al., *Anti-MOG antibodies are under polygenic regulation with the most significant control coming from the C-type lectin-like gene locus.* Genes Immun, 2013. **14**(7): p. 409-19.
127. Blankenhorn, E.P., et al., *Genetic analysis of the influence of pertussis toxin on experimental allergic encephalomyelitis susceptibility: an environmental agent can override genetic checkpoints.* J Immunol, 2000. **164**(6): p. 3420-5.
128. Chen, X., O.M. Howard, and J.J. Oppenheim, *Pertussis toxin by inducing IL-6 promotes the generation of IL-17-producing CD4 cells.* J Immunol, 2007. **178**(10): p. 6123-9.
129. Kügler, S., et al., *Pertussis toxin transiently affects barrier integrity, organelle organization and transmigration of monocytes in a human brain microvascular endothelial cell barrier model.* Cell Microbiol, 2007. **9**(3): p. 619-32.
130. Weber, M.S., et al., *Repetitive pertussis toxin promotes development of regulatory T cells and prevents central nervous system autoimmune disease.* PLoS One, 2010. **5**(12): p. e16009.
131. Stromnes, I.M. and J.M. Goverman, *Passive induction of experimental allergic encephalomyelitis.* Nat Protoc, 2006. **1**(4): p. 1952-60.
132. Ben-Nun, A., H. Wekerle, and I.R. Cohen, *The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis.* Eur J Immunol, 1981. **11**(3): p. 195-9.
133. Stromnes, I.M., et al., *Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells.* Nat Med, 2008. **14**(3): p. 337-42.
134. Domingues, H.S., et al., *Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis.* PLoS One, 2010. **5**(11): p. e15531.
135. Bettelli, E., et al., *Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis.* J Exp Med, 2003. **197**(9): p. 1073-81.
136. Mendel, I., et al., *A novel protective model against experimental allergic encephalomyelitis in mice expressing a transgenic TCR-specific for myelin oligodendrocyte glycoprotein.* J Neuroimmunol, 2004. **149**(1-2): p. 10-21.
137. Krishnamoorthy, G., A. Holz, and H. Wekerle, *Experimental models of spontaneous autoimmune disease in the central nervous system.* J Mol Med (Berl), 2007. **85**(11): p. 1161-73.
138. Axthelm, M.K., et al., *Japanese macaque encephalomyelitis: a spontaneous multiple sclerosis-like disease in a nonhuman primate.* Ann Neurol, 2011. **70**(3): p. 362-73.
139. Locatelli, G., et al., *Primary oligodendrocyte death does not elicit anti-CNS immunity.* Nat Neurosci, 2012. **15**(4): p. 543-50.
140. Kassmann, C.M., et al., *Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes.* Nat Genet, 2007. **39**(8): p. 969-76.
141. Didonna, A., *Preclinical Models of Multiple Sclerosis: Advantages and Limitations towards Better Therapies.* Curr Med Chem, 2016.
142. Krishnamoorthy, G., et al., *Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation.* J Clin Invest, 2006. **116**(9): p. 2385-92.
143. Traka, M., et al., *Oligodendrocyte death results in immune-mediated CNS demyelination.* Nat Neurosci, 2016. **19**(1): p. 65-74.
144. Ferber, I.A., et al., *Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE).* J Immunol, 1996. **156**(1): p. 5-7.
145. Gran, B., et al., *IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination.* J Immunol, 2002. **169**(12): p. 7104-10.
146. Berer, K., et al., *Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination.* Nature, 2011. **479**(7374): p. 538-41.
147. Wu, C., et al., *Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1.* Nature, 2013. **496**(7446): p. 513-7.

148. Kleinewietfeld, M., et al., *Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells.* Nature, 2013. **496**(7446): p. 518-22.
149. Baker, D., et al., *Critical appraisal of animal models of multiple sclerosis.* Mult Scler, 2011. **17**(6): p. 647-57.
150. Gregory, A.P., et al., *TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis.* Nature, 2012. **488**(7412): p. 508-11.
151. Anderton, S.M., *Post-translational modifications of self antigens: implications for autoimmunity.* Curr Opin Immunol, 2004. **16**(6): p. 753-8.
152. Doyle, H.A. and M.J. Mamula, *Posttranslational modifications of self-antigens.* Ann N Y Acad Sci, 2005. **1050**: p. 1-9.
153. Bischoff, R. and H. Schlüter, *Amino acids: chemistry, functionality and selected non-enzymatic post-translational modifications.* J Proteomics, 2012. **75**(8): p. 2275-96.
154. Uy, R. and F. Wold, *Posttranslational covalent modification of proteins.* Science, 1977. **198**(4320): p. 890-6.
155. Doyle, H.A., R.J. Gee, and M.J. Mamula, *Altered immunogenicity of isoaspartate containing proteins.* Autoimmunity, 2007. **40**(2): p. 131-7.
156. Jia, Y., et al., *Early embryonic lethality of mice with disrupted transcription cofactor PIMT/NCOA6IP/Tgs1 gene.* Mech Dev, 2012. **129**(9-12): p. 193-207.
157. Zhu, J.X., et al., *Protein repair in the brain, proteomic analysis of endogenous substrates for protein L-isoaspartyl methyltransferase in mouse brain.* J Biol Chem, 2006. **281**(44): p. 33802-13.
158. Chondrogianni, N., et al., *Protein damage, repair and proteolysis.* Mol Aspects Med, 2014. **35**: p. 1-71.
159. Jaisson, S. and P. Gillery, *Evaluation of nonenzymatic posttranslational modification-derived products as biomarkers of molecular aging of proteins.* Clin Chem, 2010. **56**(9): p. 1401-12.
160. Cusick, M.F., J.E. Libbey, and R.S. Fujinami, *Molecular mimicry as a mechanism of autoimmune disease.* Clin Rev Allergy Immunol, 2012. **42**(1): p. 102-11.
161. Canton, J., D. Neculai, and S. Grinstein, *Scavenger receptors in homeostasis and immunity.* Nat Rev Immunol, 2013. **13**(9): p. 621-34.
162. Manoury, B., et al., *Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP.* Nat Immunol, 2002. **3**(2): p. 169-74.
163. Antoniou, A.N., et al., *Control of antigen presentation by a single protease cleavage site.* Immunity, 2000. **12**(4): p. 391-8.
164. Lundberg, K., et al., *Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity.* Arthritis Res Ther, 2005. **7**(3): p. R458-67.
165. Wang, Z., et al., *Protein carbamylation links inflammation, smoking, uremia and atherogenesis.* Nat Med, 2007. **13**(10): p. 1176-84.
166. Reed, E., et al., *Antibodies to carbamylated α-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies.* Arthritis Res Ther, 2016. **18**(1): p. 96.
167. Cao, L., D. Sun, and J.N. Whitaker, *Citrullinated myelin basic protein induces experimental autoimmune encephalomyelitis in Lewis rats through a diverse T cell repertoire.* J Neuroimmunol, 1998. **88**(1-2): p. 21-9.
168. Moscarello, M.A., F.G. Mastronardi, and D.D. Wood, *The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis.* Neurochem Res, 2007. **32**(2): p. 251-6.
169. Kidd, B.A., et al., *Epitope spreading to citrullinated antigens in mouse models of autoimmune arthritis and demyelination.* Arthritis Res Ther, 2008. **10**(5): p. R119.
170. He, X.L., et al., *Structural snapshot of aberrant antigen presentation linked to autoimmunity: the immunodominant epitope of MBP complexed with I-Au.* Immunity, 2002. **17**(1): p. 83-94.
171. Fiorini, A., et al., *Involvement of oxidative stress in occurrence of relapses in multiple sclerosis: the spectrum of oxidatively modified serum proteins detected by proteomics and redox proteomics analysis.* PLoS One, 2013. **8**(6): p. e65184.
172. Ferretti, G. and T. Bacchetti, *Peroxidation of lipoproteins in multiple sclerosis.* J Neurol Sci, 2011. **311**(1-2): p. 92-7.
173. Gonzalo, H., et al., *Lipidome analysis in multiple sclerosis reveals protein lipoxidative damage as a potential pathogenic mechanism.* J Neurochem, 2012. **123**(4): p. 622-34.
174. Artemiadis, A.K. and M.C. Anagnostouli, *Apoptosis of oligodendrocytes and post-translational modifications of myelin basic protein in multiple sclerosis: possible role for the early stages of multiple sclerosis.* Eur Neurol, 2010. **63**(2): p. 65-72.
175. Papac-Milicevic, N., C.J.L. Busch, and C.J. Binder, *Malondialdehyde Epitopes as Targets of Immunity and the Implications for Atherosclerosis*, in *Advances in Immunology*. 2016, Academic Press.
176. Nam, T.G., *Lipid peroxidation and its toxicological implications.* Toxicol Res, 2011. **27**(1): p. 1-6.
177. Yin, H., L. Xu, and N.A. Porter, *Free radical lipid peroxidation: mechanisms and analysis.* Chem Rev, 2011. **111**(10): p. 5944-72.

178. Rubbo, H., et al., *Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives.* J Biol Chem, 1994. **269**(42): p. 26066-75.
179. Niki, E., *Lipid peroxidation: physiological levels and dual biological effects.* Free Radic Biol Med, 2009. **47**(5): p. 469-84.
180. Kaur, K., et al., *(Carboxyalkyl)pyrroles in human plasma and oxidized low-density lipoproteins.* Chem Res Toxicol, 1997. **10**(12): p. 1387-96.
181. Hammarström, S. and P. Falardeau, *Resolution of prostaglandin endoperoxide synthase and thromboxane synthase of human platelets.* Proc Natl Acad Sci U S A, 1977. **74**(9): p. 3691-5.
182. Sattler, S.E., et al., *Nonenzymatic lipid peroxidation reprograms gene expression and activates defense markers in Arabidopsis tocopherol-deficient mutants.* Plant Cell, 2006. **18**(12): p. 3706-20.
183. Harris, R.A. and S. Amor, *Sweet and sour--oxidative and carbonyl stress in neurological disorders.* CNS Neurol Disord Drug Targets, 2011. **10**(1): p. 82-107.
184. Gutteridge, J.M., *Lipid peroxidation and antioxidants as biomarkers of tissue damage.* Clin Chem, 1995. **41**(12 Pt 2): p. 1819-28.
185. Hecker, M. and V. Ullrich, *On the mechanism of prostacyclin and thromboxane A2 biosynthesis.* J Biol Chem, 1989. **264**(1): p. 141-50.
186. Niki, E., *Antioxidants in relation to lipid peroxidation.* Chem Phys Lipids, 1987. **44**(2-4): p. 227-53.
187. Hamelin, S.S. and A.C. Chan, *Modulation of platelet thromboxane and malonaldehyde by dietary vitamin E and linoleate.* Lipids, 1983. **18**(3): p. 267-9.
188. Ström, M., et al., *Naturally occurring genetic variability in expression of Gsta4 is associated with differential survival of axotomized rat motoneurons.* Neuromolecular Med, 2012. **14**(1): p. 15-29.
189. McCaskill, M.L., et al., *Hybrid malondialdehyde and acetaldehyde protein adducts form in the lungs of mice exposed to alcohol and cigarette smoke.* Alcohol Clin Exp Res, 2011. **35**(6): p. 1106-13.
190. Freeman, T.L., et al., *Aldehydes in cigarette smoke react with the lipid peroxidation product malonaldehyde to form fluorescent protein adducts on lysines.* Chem Res Toxicol, 2005. **18**(5): p. 817-24.
191. Villa, S., M. Livio, and G. de Gaetano, *The inhibitory effect of aspirin on platelet and vascular prostaglandins in rats cannot be completely dissociated.* Br J Haematol, 1979. **42**(3): p. 425-31.
192. Cowan, D.H., *Platelet adherence to collagen: role of prostaglandin-thromboxane synthesis.* Br J Haematol, 1981. **49**(3): p. 425-34.
193. Chehade, J., et al., *Malondialdehyde binding of rat cerebral proteins is reduced in experimental hypothyroidism.* Brain Res, 1999. **829**(1-2): p. 201-3.
194. Sirota, R., et al., *Coffee polyphenols protect human plasma from postprandial carbonyl modifications.* Mol Nutr Food Res, 2013. **57**(5): p. 916-9.
195. Cheeseman, K.H., A. Beavis, and H. Esterbauer, *Hydroxyl-radical-induced iron-catalysed degradation of 2-deoxyribose. Quantitative determination of malondialdehyde.* Biochem J, 1988. **252**(3): p. 649-53.
196. Quash, G., et al., *Malondialdehyde production from spermine by homogenates of normal and transformed cells.* Biochimie, 1987. **69**(2): p. 101-8.
197. Williams, J.D., et al., *Malondialdehyde-derived epitopes in human skin result from acute exposure to solar UV and occur in nonmelanoma skin cancer tissue.* J Photochem Photobiol B, 2014. **132**: p. 56-65.
198. Dennis, K.J. and T. Shibamoto, *Production of malonaldehyde from squalene, a major skin surface lipid, during UV-irradiation.* Photochem Photobiol, 1989. **49**(5): p. 711-6.
199. Del Giudice, G. and R. Rappuoli, *Inactivated and adjuvanted influenza vaccines.* Curr Top Microbiol Immunol, 2015. **386**: p. 151-80.
200. O'Hagan, D.T., et al., *The mechanism of action of MF59 - an innately attractive adjuvant formulation.* Vaccine, 2012. **30**(29): p. 4341-8.
201. Marnett, L.J., et al., *Studies of the hydrolysis of 14C-labeled tetraethoxypropane to malondialdehyde.* Anal Biochem, 1979. **99**(2): p. 458-63.
202. Esterbauer, H., R.J. Schaur, and H. Zollner, *Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes.* Free Radic Biol Med, 1991. **11**(1): p. 81-128.
203. Uchida, K., et al., *Protein modification by lipid peroxidation products: formation of malondialdehyde-derived N(epsilon)-(2-propenol)lysine in proteins.* Arch Biochem Biophys, 1997. **346**(1): p. 45-52.
204. Zhao, J., et al., *Mass spectrometric evidence of malonaldehyde and 4-hydroxynonenal adductions to radical-scavenging soy peptides.* J Agric Food Chem, 2012. **60**(38): p. 9727-36.
205. Draper, H.H. and M. Hadley, *A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde.* Xenobiotica, 1990. **20**(9): p. 901-7.
206. Cordes, E. and W. Jencks, *The Mechanism of Hydrolysis of Schiff Bases Derived from Aliphatic Amines.* Journal of the American Chemical Society, 1963.
207. Bubnis, W.A. and C.M. Ofner, *The determination of epsilon-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid.* Anal Biochem, 1992. **207**(1): p. 129-33.

208. Xu, D., et al., *Epitope characterization of malondialdehyde-acetaldehyde adducts using an enzyme-linked immunosorbent assay.* Chem Res Toxicol, 1997. **10**(9): p. 978-86.
209. Ishii, T., et al., *Mass spectroscopic characterization of protein modification by malondialdehyde.* Chem Res Toxicol, 2006. **19**(1): p. 122-9.
210. Tuma, D.J., et al., *Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration.* Hepatology, 1996. **23**(4): p. 872-80.
211. Itakura, K., K. Uchida, and T. Osawa, *A novel fluorescent malondialdehyde-lysine adduct.* Chemistry and Physics of Lipids, 1996. **84**: p. 75-79
212. Uchida, K., *Lipofuscin-like fluorophores originated from malondialdehyde.* Free Radic Res, 2006. **40**(12): p. 1335-8.
213. Mooradian, A.D., et al., *Malondialdehyde modification of proteins in vitro is enhanced in the presence of acetaldehyde.* Nutrition, 2001. **17**(7-8): p. 619-22.
214. Slatter, D.A., M. Murray, and A.J. Bailey, *Formation of a dihydropyridine derivative as a potential cross-link derived from malondialdehyde in physiological systems.* FEBS Lett, 1998. **421**(3): p. 180-4.
215. Tuma, D.J., et al., *Elucidation of reaction scheme describing malondialdehyde-acetaldehyde-protein adduct formation.* Chem Res Toxicol, 2001. **14**(7): p. 822-32.
216. Millanta, S., et al., *Short exposure of albumin to high concentrations of malondialdehyde does not mimic physiological conditions.* Exp Mol Pathol, 2013. **94**(1): p. 270-6.
217. Gómez-Sánchez, A., I. Hermosín, and I. Maya, *Cleavage and oligomerization of malondialdehyde under physiological conditions.* Tetrahedron Letters, 1990.
218. Gómez-Sánchez, A., et al., *Cleavage and oligomerization of malondialdehyde.* Tetrahedron, 1993. **49**(6): p. 1237-1250.
219. Gutteridge, J.M., *The use of standards for malonyldialdehyde.* Anal Biochem, 1975. **69**(2): p. 518-26.
220. Gutteridge, J.M.C., A.D. Heys, and J. Lunec, *Fluorescent malondialdehyde polymers from hydrolysed 1,1,3,3-tetramethoxypropane.* Analytica Chimica Acta, 1977. **94**(1): p. 209-211.
221. Willis, M.S., et al., *T cell proliferative responses to malondialdehyde-acetaldehyde haptenated protein are scavenger receptor mediated.* Int Immunopharmacol, 2003. **3**(10-11): p. 1381-99.
222. Kwon, H.J., et al., *Aldehyde dehydrogenase 2 deficiency ameliorates alcoholic fatty liver but worsens liver inflammation and fibrosis in mice.* Hepatology, 2014. **60**(1): p. 146-57.
223. Marnett, L.J., et al., *Distribution and oxidation of malondialdehyde in mice.* Prostaglandins, 1985. **30**(2): p. 241-54.
224. Siu, G.M. and H.H. Draper, *Metabolism of malonaldehyde in vivo and in vitro.* Lipids, 1982. **17**(5): p. 349-55.
225. Kapphahn, R.J., et al., *Retinal proteins modified by 4-hydroxynonenal: identification of molecular targets.* Exp Eye Res, 2006. **83**(1): p. 165-75.
226. Pizzimenti, S., et al., *Interaction of aldehydes derived from lipid peroxidation and membrane proteins.* Front Physiol, 2013. **4**: p. 242.
227. Hyvärinen, S., et al., *Recognition of malondialdehyde-modified proteins by the C terminus of complement factor H is mediated via the polyanion binding site and impaired by mutations found in atypical hemolytic uremic syndrome.* J Biol Chem, 2014. **289**(7): p. 4295-306.
228. Gros, P., F.J. Milder, and B.J. Janssen, *Complement driven by conformational changes.* Nat Rev Immunol, 2008. **8**(1): p. 48-58.
229. Wang, F.M., et al., *The dysfunctions of complement factor H in lupus nephritis.* Lupus, 2016.
230. Zarkovic, N., et al., *Pathophysiological relevance of aldehydic protein modifications.* J Proteomics, 2013. **92**: p. 239-47.
231. Tuma, D.J., *Role of malondialdehyde-acetaldehyde adducts in liver injury.* Free Radic Biol Med, 2002. **32**(4): p. 303-8.
232. Wyatt, T.A., et al., *Malondialdehyde-acetaldehyde-adducted protein inhalation causes lung injury.* Alcohol, 2012. **46**(1): p. 51-9.
233. López, J., et al., *Oxidative stress markers in surgically treated patients with refractory epilepsy.* Clin Biochem, 2007. **40**(5-6): p. 292-8.
234. Uchida, K., *Role of reactive aldehyde in cardiovascular diseases.* Free Radic Biol Med, 2000. **28**(12): p. 1685-96.
235. Antoniak, D.T., et al., *Aldehyde-modified proteins as mediators of early inflammation in atherosclerotic disease.* Free Radic Biol Med, 2015. **89**: p. 409-18.
236. Kaida, H., et al., *Positive correlation between malondialdehyde-modified low-density lipoprotein cholesterol and vascular inflammation evaluated by 18F-FDG PET/CT.* Atherosclerosis, 2014. **237**(2): p. 404-9.
237. Feng, B., et al., *The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages.* Nat Cell Biol, 2003. **5**(9): p. 781-92.
238. Riggins, J.N. and L.J. Marnett, *Mutagenicity of the malondialdehyde oligomerization products 2-(3'-oxo-1'-propenyl)-malondialdehyde and 2,4-dihydroxymethylene-3-(2,2-dimethoxyethyl)glutaraldehyde in Salmonella.* Mutat Res, 2001. **497**(1-2): p. 153-7.

239. Plastaras, J.P., et al., *Reactivity and mutagenicity of endogenous DNA oxopropenylating agents: base propenals, malondialdehyde, and N(epsilon)-oxopropenyllysine.* Chem Res Toxicol, 2000. **13**(12): p. 1235-42.
240. Cline, S.D., et al., *Malondialdehyde adducts in DNA arrest transcription by T7 RNA polymerase and mammalian RNA polymerase II.* Proc Natl Acad Sci U S A, 2004. **101**(19): p. 7275-80.
241. Shanmugam, N., et al., *Proinflammatory effects of advanced lipoxidation end products in monocytes.* Diabetes, 2008. **57**(4): p. 879-88.
242. Raghavan, S., G. Subramaniyam, and N. Shanmugam, *Proinflammatory effects of malondialdehyde in lymphocytes.* J Leukoc Biol, 2012. **92**(5): p. 1055-67.
243. Duryee, M.J., et al., *Lipopolysaccharide is a cofactor for malondialdehyde-acetaldehyde adduct-mediated cytokine/chemokine release by rat sinusoidal liver endothelial and Kupffer cells.* Alcohol Clin Exp Res, 2004. **28**(12): p. 1931-8.
244. Kharbanda, K.K., et al., *Malondialdehyde-acetaldehyde-protein adducts increase secretion of chemokines by rat hepatic stellate cells.* Alcohol, 2001. **25**(2): p. 123-8.
245. Kikugawa, K., H. Kosugi, and T. Asakura, *Effect of malondialdehyde, a product of lipid peroxidation, on the function and stability of hemoglobin.* Arch Biochem Biophys, 1984. **229**(1): p. 7-14.
246. Thiele, G.M., et al., *Malondialdehyde-acetaldehyde (MAA) modified proteins induce pro-inflammatory and pro-fibrotic responses by liver endothelial cells.* Comp Hepatol, 2004. **3 Suppl 1**: p. S25.
247. Hill, G.E., et al., *Association of malondialdehyde-acetaldehyde (MAA) adducted proteins with atherosclerotic-induced vascular inflammatory injury.* Atherosclerosis, 1998. **141**(1): p. 107-16.
248. Willis, M.S., et al., *Malondialdehyde-acetaldehyde haptenated protein binds macrophage scavenger receptor(s) and induces lysosomal damage.* Int Immunopharmacol, 2004. **4**(7): p. 885-99.
249. Kaemmerer, E., et al., *Effects of lipid peroxidation-related protein modifications on RPE lysosomal functions and POS phagocytosis.* Invest Ophthalmol Vis Sci, 2007. **48**(3): p. 1342-7.
250. Klassen, L.W., et al., *Conversion of acetaldehyde-protein adduct epitopes from a nonreduced to a reduced phenotype by antigen processing cells.* Alcohol Clin Exp Res, 1999. **23**(4): p. 657-63.
251. Kharbanda, K.K., et al., *Effect of malondialdehyde-acetaldehyde-protein adducts on the protein kinase C-dependent secretion of urokinase-type plasminogen activator in hepatic stellate cells.* Biochem Pharmacol, 2002. **63**(3): p. 553-62.
252. Greig, F.H., S. Kennedy, and C.M. Spickett, *Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation.* Free Radic Biol Med, 2012. **52**(2): p. 266-80.
253. Ott, C., et al., *Role of advanced glycation end products in cellular signaling.* Redox Biol, 2014. **2**: p. 411-29.
254. Thiele, G.M., et al., *The chemistry and biological effects of malondialdehyde-acetaldehyde adducts.* Alcohol Clin Exp Res, 2001. **25**(5 Suppl ISBRA): p. 218S-224S.
255. Weismann, D. and C.J. Binder, *The innate immune response to products of phospholipid peroxidation.* Biochim Biophys Acta, 2012. **1818**(10): p. 2465-75.
256. Miller, Y.I., et al., *Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity.* Circ Res, 2011. **108**(2): p. 235-48.
257. Berger, J.P., et al., *Malondialdehyde-acetaldehyde (MAA) adducted proteins bind to scavenger receptor A in airway epithelial cells.* Alcohol, 2014. **48**(5): p. 493-500.
258. Shechter, I., et al., *The metabolism of native and malondialdehyde-altered low density lipoproteins by human monocyte-macrophages.* J Lipid Res, 1981. **22**(1): p. 63-71.
259. Nicoletti, A., et al., *The macrophage scavenger receptor type A directs modified proteins to antigen presentation.* Eur J Immunol, 1999. **29**(2): p. 512-21.
260. Duryee, M.J., et al., *Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins.* Mol Pharmacol, 2005. **68**(5): p. 1423-30.
261. Zhu, X., et al., *Scavenger receptor function of mouse Fcγ receptor III contributes to progression of atherosclerosis in apolipoprotein E hyperlipidemic mice.* J Immunol, 2014. **193**(5): p. 2483-95.
262. Duryee, M.J., et al., *Malondialdehyde-acetaldehyde adduct is the dominant epitope after MDA modification of proteins in atherosclerosis.* Free Radic Biol Med, 2010. **49**(10): p. 1480-6.
263. Heine, G.H., et al., *Monocyte subpopulations and cardiovascular risk in chronic kidney disease.* Nat Rev Nephrol, 2012. **8**(6): p. 362-9.
264. Abdul-Majid, K.B., et al., *Fc receptors are critical for autoimmune inflammatory damage to the central nervous system in experimental autoimmune encephalomyelitis.* Scand J Immunol, 2002. **55**(1): p. 70-81.
265. Breij, E.C., et al., *The FcRgamma chain is not essential for induction of experimental allergic encephalomyelitis (EAE) or anti-myelin antibody-mediated exacerbation of EAE.* J Neuropathol Exp Neurol, 2005. **64**(4): p. 304-11.
266. Nickel, T., et al., *oxLDL uptake by dendritic cells induces upregulation of scavenger-receptors, maturation and differentiation.* Atherosclerosis, 2009. **205**(2): p. 442-50.
267. Chou, M.Y., et al., *Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans.* J Clin Invest, 2009. **119**(5): p. 1335-49.

268. Tsiantoulas, D., et al., *Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies.* J Lipid Res, 2015. **56**(2): p. 440-8.
269. Wang, C., et al., *Natural antibodies of newborns recognize oxidative stress-related malondialdehyde acetaldehyde adducts on apoptotic cells and atherosclerotic plaques.* Int Immunol, 2013. **25**(10): p. 575-87.
270. Chipinda, I., J.M. Hettick, and P.D. Siegel, *Haptenation: chemical reactivity and protein binding.* J Allergy (Cairo), 2011. **2011**: p. 839682.
271. Wuttge, D.M., M. Bruzelius, and S. Stemme, *T-cell recognition of lipid peroxidation products breaks tolerance to self proteins.* Immunology, 1999. **98**(2): p. 273-9.
272. Wållberg, M., et al., *Malondialdehyde modification of myelin oligodendrocyte glycoprotein leads to increased immunogenicity and encephalitogenicity.* Eur J Immunol, 2007. **37**(7): p. 1986-95.
273. Willis, M.S., et al., *Adduction of soluble proteins with malondialdehyde-acetaldehyde (MAA) induces antibody production and enhances T-cell proliferation.* Alcohol Clin Exp Res, 2002. **26**(1): p. 94-106.
274. Palinski, W., et al., *ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum.* Arterioscler Thromb, 1994. **14**(4): p. 605-16.
275. Duryee, M.J., et al., *Increased immunogenicity to P815 cells modified with malondialdehyde and acetaldehyde.* Int Immunopharmacol, 2008. **8**(8): p. 1112-8.
276. Thiele, G.M., et al., *Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant.* Alcohol Clin Exp Res, 1998. **22**(8): p. 1731-9.
277. Schwartz, M. and C. Raposo, *Protective Autoimmunity: A Unifying Model for the Immune Network Involved in CNS Repair.* Neuroscientist, 2014. **20**(4): p. 343-358.
278. Palinski, W., E. Miller, and J.L. Witztum, *Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis.* Proc Natl Acad Sci U S A, 1995. **92**(3): p. 821-5.
279. Freigang, S., et al., *Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes.* Arterioscler Thromb Vasc Biol, 1998. **18**(12): p. 1972-82.
280. Schiopu, A., et al., *Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis.* Circulation, 2004. **110**(14): p. 2047-52.
281. Fredrikson, G.N., et al., *Atheroprotective immunization with MDA-modified apo B-100 peptide sequences is associated with activation of Th2 specific antibody expression.* Autoimmunity, 2005. **38**(2): p. 171-9.
282. Ylä-Herttuala, S., et al., *Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man.* J Clin Invest, 1989. **84**(4): p. 1086-95.
283. Maiolino, G., et al., *Antibodies to malondialdehyde oxidized low-density lipoproteins predict long term cardiovascular mortality in high risk patients.* Int J Cardiol, 2013. **168**(1): p. 484-9.
284. Amir, S., et al., *Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease.* J Lipid Res, 2012. **53**(7): p. 1316-26.
285. Zhou, X., et al., *Lesion development and response to immunization reveal a complex role for CD4 in atherosclerosis.* Circ Res, 2005. **96**(4): p. 427-34.
286. Binder, C.J., et al., *IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis.* J Clin Invest, 2004. **114**(3): p. 427-37.
287. Richter, T., et al., *Immunochemical crossreactivity of antibodies specific for "advanced glycation endproducts" with "advanced lipoxidation endproducts".* Neurobiol Aging, 2005. **26**(4): p. 465-74.
288. Binder, C.J., et al., *Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL.* Nat Med, 2003. **9**(6): p. 736-43.
289. Wållberg, M., J. Wefer, and R.A. Harris, *Vaccination with myelin oligodendrocyte glycoprotein adsorbed to alum effectively protects DBA/1 mice from experimental autoimmune encephalomyelitis.* Eur J Immunol, 2003. **33**(6): p. 1539-47.
290. Amor, S., et al., *Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice.* J Immunol, 1994. **153**(10): p. 4349-56.
291. Mia, S., et al., *An optimized protocol for human M2 macrophages using M-CSF and IL-4/IL-10/TGF-β yields a dominant immunosuppressive phenotype.* Scand J Immunol, 2014. **79**(5): p. 305-14.
292. Xue, J., et al., *Transcriptome-based network analysis reveals a spectrum model of human macrophage activation.* Immunity, 2014. **40**(2): p. 274-88.
293. Perdiguero, E.G. and F. Geissmann, *The development and maintenance of resident macrophages.* Nat Immunol, 2016. **17**(1): p. 2-8.
294. Okabe, Y. and R. Medzhitov, *Tissue biology perspective on macrophages.* Nat Immunol, 2016. **17**(1): p. 9-17.

295. Goldmann, T., et al., *Origin, fate and dynamics of macrophages at central nervous system interfaces.* Nat Immunol, 2016.
296. O'Boyle, N.M., et al., *Open Babel: An open chemical toolbox.* J Cheminform, 2011. **3**: p. 33.
297. Baker, N.A., et al., *Electrostatics of nanosystems: application to microtubules and the ribosome.* Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10037-41.
298. Pronk, S., et al., *GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit.* Bioinformatics, 2013. **29**(7): p. 845-54.
299. Phillips, J.C., et al., *Scalable molecular dynamics with NAMD.* J Comput Chem, 2005. **26**(16): p. 1781-802.
300. Ponder, J.W. and D.A. Case, *Force fields for protein simulations.* Adv Protein Chem, 2003. **66**: p. 27-85.
301. Carrillo-Vico, A., M.D. Leech, and S.M. Anderton, *Contribution of myelin autoantigen citrullination to T cell autoaggression in the central nervous system.* J Immunol, 2010. **184**(6): p. 2839-46.